IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	:	
Giulio ALESSANDRI et al.	:	Art Unit: 1651
Serial No.: 10/510,622	:	Examiner: Bernhart, Lora Elizabeth
Filed: 06/27/2005	:	

For: A PROCESS FOR THE PREPARATION OF STEM CELLS FROM HUMAN MUSCLE TISSUE AND ADIPOSE TISSUE, AND STEM CELLS OBTAINABLE BY THIS PROCESS

DECLARATION

I, Giulio Alessandri, hereby declare the following:

1. My curriculum vitae is attached as annex 1 to the present declaration; I am a co-inventor of the above-identified patent application.

2. In a first Office Action and a second (final) Office Action issued by the U.S. Patent and Trademark Office, the Examiner in charge of the above-identified patent application contended that the claims under examination were not enabled by the specification as originally filed, since the specification does not disclose how the claimed process applies to cells obtained from the adipose tissue. Moreover, the Examiner points out that the working example in the specification does not include a characterization of the hFSCs obtainable form adipose tissue.

3. Attached herewith, as annex 2, is an experimental report illustrating the functional characterization of the human fat stem cells (hFSCs) obtainable by the process of the invention In particular, the experimental report illustrates the differentiation conditions applied to both human fat stem cells (hFSCs) and human muscle stem cells (hMSCs) in order to obtain the differentiation of both stem cell types into astrocytes and neurones (neurogenic differentiation), bone cells (osteogenic differentiation), endothelial cells (vasculogenic differentiation) and muscle cells (myogenic differentiation). The process for obtaining the hFSCs from adipose tissue is described

at pages 8-9 of the patent specification.

4. The experiments in the experimental report were carried out at the laboratory of Microbiology (University of Brescia, Brescia, Italy) and Medestrea Research Laboratories (Consorzio Carso (Gerenzano, Bari, Italy). I have personally supervised the experiments, reviewed the data obtained there from and prepared the annexed report.

5. I hereby certify that the report accurately summarizes the experimental procedures and the results obtained from the experimental study. The following general conclusion can be drawn from the obtained results:
That, by applying the isolation process disclosed at pages 8-9 of the patent specification, human fat stem cells (hFSCs) are obtained from human adipose tissue. That the obtained hFSCs are capable differentiating into nerve cells, vascular cells, muscle cells and bone cells under the same culture conditions which are required for the differentiation of human muscle stem cells (hMSCs) into the same cell type.
The differentiation of hMSCs into nerve cells, bone cells, muscle cells and endothelial cells is disclosed in the patent specification, particularly in the working example at pages 10-11. Some details relating to the composition of the osteogenic standard medium and endothelial differentiation standard medium are not explicitly mentioned in the working example; however, these differentiation media were standard media, the compositions of which were known to the person skilled in the art before the filing date of the application.
It is therefore concluded that the technical teachings contained in the patent specification, combined with the common general knowledge available in the art before the filing of the patent application particularly with respect to the composition of standard osteogenic and endothelial differentiation media, are sufficient to enable the person skilled in the art to carry out the invention, that is to say: 1) to obtain hFSCs from human adipose tissue; and 2) to differentiate the obtained hFSCs into nerve cells, vascular cells and bone cells.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these were made with the knowledge that false statements made willfully are punishable by fine, imprisonment, or both a fine and imprisonment under Section 1001 of Title 18 of the United States; and further that false statements made willfully may jeopardize the validity of any patent issuing on a patent application in which the false

statements were made.

Annex 1: Curriculum Vitae

Annex 2: Experimental report

Date: 12-1-08

Giulio Alessandri

Giulio Alessandri
Curriculum vitae

Dr. Giulio Alessandri was born in Milan (Italy) on 4 March 1951. He obtained his Master in Pharmacological Research from the Institute of Pharmacological Research "Mario Negri" in 1975 and his ph.D in Biology from the University of Milan in 1980.

Scientific career:

1974-1976: **Special student**, Laboratory of Immunology and Cancer Chemiotherapy, Institute of Pharmacological Research "Mario Negri", Milan, Italy

1977-1980 (August): **Research Assistant**, Laboratory of Human Immunology, Institute of Pharmacological Research "Mario Negri", Milan, Italy

1980 (September) - 1983: **Visiting Fellow**, Pathophysiology Department, DHHS, National Institute of Health, National Cancer Institute (NCI), Bethesda, Md. USA

1984 (April) -1985 (May): **Visiting Associate**, Laboratory of Cell Immunology, DHHS, National Institute of Health, National Cancer Institute (NCI), Bethesda, Md. USA

1986-1987: **Project Leader**, Laboratory of Vascular Biology, Farmitalia, "Carlo Erba" S.p.A, Milan, Italy

1988-1993: **Scientific Consultant**, Laboratory of Cell Biology, FIDIA, S.p.A., Abano Terme (Padova) Italy. **Project Leader**, Dipartment of Human Oncology, University of Turin and San Giovanni antica sede Hospital Turin, Italy

1994-1998 (March): **Scientific Consultant**, Laboratory of Cell Biology Italfarmaco S.p.A. Centro Ricerche Cinisello Balsamo, Milan, Project Leader, Laboratory of Cell Biology National Cancer Institute of Genova and Laboratory of Microbiology, "Giannina Gaslini" Institute of Genova; Italy

1998 (April) - 2003 (January): **Research Leader**, Laboratory of Microbiology, Department of Experimental Medicine, University of Brescia; **Scientific Consultant**, Laboratory of Oncology, Research Centre, Upjonh Farmacia, Nerviano (Milan), **Scientific Consultant** of Medestea International S.p.A, Laboratory of Human Stem Cells Biopark Ivrea, Turin, Italy.

2003 (June – October) **Visiting Scientist**, Department of Pathology, VA Hospital and University of Washington, WA, USA

2003 (February) - today: **Research Leader**, SSD-UCV Laboratory of Neurobiology and Neuroregenerative Therapies, Neurological Institute " Carlo Besta", Milan Italy.

Scientific interests

During the last 20 years Dr. Alessandri has carried out an intense research activity. In particular he has been interested in .angiogenesis. He developed method for isolation and culture microvascular endothelial cells. Particularly he was able to isolate endothelial cells derived from neoplastic tissues. He found that molecules released by tumor cells may stimulate tumor angiogenesis and facilitate metastatic formation. During this activity he collaborated with several international scientists particularly in USA that included Prof PM Gullino (that was his tutor at NIH, NCI during his stage in USA), Rakesh Jane (Harvard University) and the recently deceased Judah Folkman, who was the pioneer in the angiogenesis research field. He is still collaborating with important researchers in the angiogenesis field. More specifically together with Prof Roberto Nicosia at Washington University (Seattle USA), using an ex in vivo method to study vessels formation (ring aorta assay), demonstrated that human fetal aorta contains immature cells able to regenerate blood vessels. Actually Dr. Alessandri and Prof Nicosia are studying the role of specific cells named "Vascular progenitors" in the formation of new vessels. Concomitantly Dr. Alessandri as Research Leader at Neurological Institute " Carlo Besta", has been involved in the isolation and identification of mesenchymal stem cells from human adult tissue. He has recently identified a population of immature cells of mesenchymal origin from human skeletal muscle that was able to differentiate into several cell lineages including nervous cells. Dr. Alessandri is author of more of 80 pear review international papers in the field of Angiogenesis and Stem cells research.

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Annex 2 to the declaration of Giulio Alessandri

EXPERIMENTAL REPORT

Assessment of the differentiation abilities of human fat stem cells (hFSCs) and human muscle stem cells (hMSC)

By

Dr. Giulio Alessandri

Milan, November 19, 2008

Isolation of hFSCs

Human Fat Stem Cells (hFSCs) were obtained as disclosed on pages 8-9 of U.S. Patent Application Serial No. 10/510,622.

Differentiation of hFSCs

The hFSCs obtained as disclosed above were cultured under various differentiation conditions in order to show their differentiation abilities.

The following differentiation conditions were applied:

Neurogenic differentiation

hFSCs were cultured on laminin matrix in the presence of neurogenic differentiating medium, consisting of the culture medium used for isolation of hFSCs but without growth factors. After 10-14 days of incubation, the expression of GFAP (astrocyte marker), β-tub III (neurone marker), Gal-C (oligodendrocyte marker) and GAD (GABAergic marker) was investigated. The results are provided below.

Osteogenic differentiation

The ability of hFSCs to form bone cells (osteoblasts) was assessed by incubating the cells for 18-21 days in osteogenic standard medium. The osteogenic standard medium is DMEM/F12 plus dexamethasone, ascorbate-2-phosphate and β-glicerophosphate (Jaiswal N, Haynesworth S E, Caplan A I, Bruder S P. Osteogenic Differentiation of Purified, Culture-Expanded Human Mesenchymal Stem Cells In Vitro. J. Cell. Biochem. 64:295-312(1997), enclosed as **Annex 3**). The expression of the proteins osteocalcin and alkaline phosphatase, which are specifically produced by osteoblasts, was assessed. The results are provided below.

Vasculogenic differentiation

hFSCs were tested for their ability to differentiate into vascular cells. The cells were cultured in 25cm2 flasks coated with fibronectin matrix for 14-20 days in a standard endothelial differentiation medium consisting of endothelial basal medium (EBM) supplemented with 10% FCS and endothelial cell growth supplement (Alessandri G, Girelli M, Taccagni G, Colombo A, Nicosia R, Caruso A, Baronio M, Pagano S, Cova L, Parati E.. Human vasculogenesis ex vivo: embryonal aorta as a tool for isolation of endothelial cell progenitors. Lab Invest. 2001 Jun;81(6):875-85, copy enclosed as **Annex 4**). At the end of the incubation, the cells were analyzed by FACS for the expression of the endothelial markers CD31, vWf, CD146 and CD36. The results are pro-

vided below.

Myogenic Differentiation

The differentiation of hFSCs to form smooth and striated muscle cells was carried out on collagen type I matrix in the presence of standard medium (DMEM/F12) without growth factors and with a low amount of FCS (3%). After 14-21 days of culture under these conditions, the expression of desmin, which is a marker for both smooth and striated muscle, and the expression of α-SMA (α-smooth muscle actine) were assessed. The results are provided below.

Differentiation of hMSCs

The differentiation conditions described above in connection with hFSCs were employed *mutatis mutandis* on human muscle stem cells (hMSCs) obtained by the isolation process disclosed on pages 5-8 of U.S. Patent Application Serial No. 10/510,622. The results obtained with hMSCs were compared with the results obtained with hFSCs.

Results

Neurogenic differentiation

hFSCs subjected to the disclosed differentiation conditions were found to express GFAP and GAD like hMSCs, indicating their ability to differentiate into astrocytes and neurones of the peripheral nervous system. After approximately 3 weeks of incubation, hFSCs were also found to express neurofilaments-M (NFM) but, similar to hMSCs, only to a limited extent.

Osteogenic differentiation

Both hFSCs and hMSCs subjected to osteogenic differentiation were found to express osteocalcin and alkaline phosphatase, indicating the ability of these cells to differentiate into bone cells.

Vasculogenic differentiation

Similar to hMSCs, hFSCs were found to express the endothelial markers CD31, vWf, CD146 and CD36 within a range of 5-14% of the total number of cells investigated, indicating their ability to undergo vasculogenic (endothelial) differentiation.

Myogenic Differentiation

Similar to hMSCs, hFSCs were found to express a significant amount of desmin as well as α-smooth muscle actine (α-SMA), indicating their ability to differentiate into myogenic cell lineages.

Journal of Cellular Biochemistry 64:295–312 (1997)

Osteogenic Differentiation of Purified, Culture-Expanded Human Mesenchymal Stem Cells In Vitro

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Abstract Human bone marrow contains a population of cells capable of differentiating along multiple mesenchymal cell lineages. Recently, techniques for the purification and culture-expansion of these human marrow-derived Mesenchymal Stem Cells (MSCs) have been developed. The goals of the current study were to establish a reproducible system for the in vitro osteogenic differentiation of human MSCs, and to characterize the effect of changes in the microenvironment upon the process. MSCs derived from 2nd or 3rd passage were cultured for 16 days in various base media containing 1 to 1000 nM dexamethasone (Dex), 0.01 to 4 mM L-ascorbic acid-2-phosphate (AsAP) or 0.25 mM ascorbic acid, and 1 to 10 mM β-glycerophosphate (βGP). Optimal osteogenic differentiation, as determined by osteoblastic morphology, expression of alkaline phosphatase (APase), reactivity with anti-osteogenic cell surface monoclonal antibodies, modulation of osteocalcin mRNA production, and the formation of a mineralized extracellular matrix containing hydroxyapatite was achieved with DMEM base medium plus 100 nM Dex, 0.05 mM AsAP, and 10 mM βGP. The formation of a continuously interconnected network of APase-positive cells and mineralized matrix supports the characterization of this progenitor population as homogeneous. While higher initial seeding densities did not affect cell number or APase activity, significantly more mineral was deposited in these cultures, suggesting that events which occur early in the differentiation process are linked to end-stage phenotypic expression. Furthermore, cultures allowed to concentrate their soluble products in the media produced more mineralized matrix, thereby implying a role for autocrine or paracrine factors synthesized by human MSCs undergoing osteoblastic lineage progression. This culture system is responsive to subtle manipulations including the basal nutrient medium, dose of physiologic supplements, cell seeding density, and volume of tissue culture medium. Cultured human MSCs provide a useful model for evaluating the multiple factors responsible for the step-wise progression of cells from undifferentiated precursors to secretory osteoblasts, and eventually terminally differentiated osteocytes. J. Cell. Biochem. 64:295–312. © 1997 Wiley-Liss, Inc.

Key words: osteoblast; glucocorticoids; hydroxyapatite; osteoprogenitor; bone marrow

Bone marrow is a complex tissue comprised of hematopoietic precursors, their differentiated progeny, and a connective tissue network referred to as stroma. The stroma itself is a heterogeneous mixture of cells including adipocytes, reticulocytes, endothelial cells, and fibroblastic cells which are in direct contact with the hematopoietic elements. Since it has been well established that the stroma contains cells that differentiate into bone, cartilage, fat, and a connective tissue which supports the differentiation of hematopoietic stem cells [Dexter and Testa, 1976; Bab et al., 1986; Beresford, 1989; Bruder et al., 1990; Bennett et al., 1991; Beresford et al., 1992], identification of the progenitor cells for these mesenchymal tissues has been an area of active investigation. Friedenstein [1976] and others [Ashton et al., 1980; Owen and Friedenstein, 1988; Beresford, 1989] have demonstrated that culture-adherent cells present in the marrow stroma are capable of differentiating into bone and cartilage when placed into an appropriate environment in vivo. These experiments have led to the hypothesis that stroma contains a unique population of stem cells which are capable of differentiating

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along multiple mesenchymal cell lineages [Owen, 1985; Owen, 1988; Beresford, 1989; Caplan, 1991]. The precedent for the existence of such a stem cell is drawn from studies of hematopoietic stem cells [Sachs, 1987], which have the capacity for self-renewal and can, under appropriate conditions, give rise to all of the hematopoietic cell types.

Recently, a series of experiments have been performed that demonstrate the existence of marrow-derived progenitors that give rise to bone [Beresford, 1989; Haynesworth et al., 1992b], cartilage [Wakitani et al., 1994; Lennon et al., 1996], muscle [Saito et al., 1995], tendon [Caplan et al., 1993], fat [Beresford et al., 1992; Dennis and Caplan, 1996], and a mature stromal phenotype that supports hematopoietic differentiation [Majumdar et al., 1995]. These cells are referred to as Mesenchymal Stem Cells (MSCs) [Owen, 1985; Caplan, 1991]. Techniques and conditions that select for these cells in culture have been established for chicken [Nakahara et al., 1990], mouse [Bergman et al., 1996; Dennis and Caplan, 1996], rat [Dennis et al., 1992], rabbit [Wakitani et al., 1994], and canine [Kadiyala et al., 1997] sources. Similarly, a more refined technique for the isolation and extensive subcultivation of human MSCs has been developed, along with a series of monoclonal antibody probes which react with the surface of human MSCs both in situ and in vitro [Haynesworth et al., 1992a,b]. Although conditions for differentiation into every mesenchymal cell lineage have not been established for every species studied, human MSCs have been serially passaged without lineage progression, and subsequently shown to form cartilage [Johnstone et al., 1996; Lennon et al., 1996], bone [Haynesworth et al., 1992b; Bruder et al., 1997], fat [Pittenger et al., 1996], and a mature stromal phenotype which supports hematopoietic differentiation in vitro [Majumdar et al., 1995]. Evidence for myogenic and tendonogenic differentiation is based on data from rat and rabbit MSCs which were either implanted in vivo in syngeneic hosts [Caplan et al., 1993; Saito et al., 1995], or treated with specific bioactive factors in vitro [Wakitani et al., 1995]. In summary, data indicate that human MSCs are capable of differentiating in vitro, at the minimum, into the bone, cartilage, fat, and mature stromal cell lineages. While adult human marrow-derived MSCs have the ability to develop into multiple mesenchymal tissue types, it is not known whether these cells are identical to stem cells residing in the mesodermal layer of the trilaminar embryo, or whether this homogeneous population represents postnatal mesenchymal progenitor cells with specific, multilineage developmental potential.

In vitro osteogenic differentiation of marrow-derived stromal cells from chick [Kamalia et al., 1992], mouse [Schoeters et al., 1988], rat [Maniatopolous et al., 1988], rabbit [Howlett et al., 1986], and pig [Thomson et al., 1993] has been reported to occur in response to various bioactive factors including osteogenin [Vukicevic et al., 1989], BMP-2 [Rickard et al., 1994], osteogenic growth peptide [Robinson et al., 1995], and the synthetic glucocorticoid dexamethasone (Dex) [Howlett et al., 1986; Maniatopoulos et al., 1988; Schoeters et al., 1988; LeBoy et al., 1991; Kamalia et al., 1992]. Although the osteochondral potential of these animal cells has been examined, little is known about the biochemical and molecular phenotype of the starting cell populations. By contrast, purified human marrow-derived MSCs have been extensively characterized with respect to their complement of cell surface and extracellular matrix molecules, as well as their secretory cytokine profile in control and experimental conditions [Haynesworth et al., 1992a, 1995, 1996]. Since endogenous systemic glucocorticoids are involved in the bone formation-bone remodeling axis [Baylink, 1983], and marrow-derived stem cells are believed to be the source of osteoblasts in the postnatal organism [Owen, 1985], Dex is a useful reagent for studies of cellular and physiologic responses. For these reasons, Dex's activity as an inductive agent in osteogenic culture systems is relevant. Therefore, the goals of the current study were to establish and characterize a reproducible system for in vitro osteogenic differentiation of purified, culture-expanded human MSCs, and to evaluate the effect of glucocorticoids upon the process. By systematically manipulating multiple variables within the tissue culture environment, we report on the phenomenon of glucocorticoid-induced osteogenic differentiation of human MSCs. This study further provides a quantitative benchmark for several parameters of osteogenesis which can be used to analyze the individual steps of osteogenic differentiation.

MATERIALS AND METHODS

Materials

Dexamethasone (Dex), sodium β-glycerophosphate (βGP), ascorbic acid (AsA), Percoll, antibi-

otic penicillin/streptomycin, alkaline phosphatase diagnostic kit #85, and calcium diagnostic kit #587 were purchased from Sigma Chemical Co. (St. Louis, MO). DMEM-LG (DMEM), α-MEM, Hams F-12, and BGJ_b media were purchased from GIBCO (Grand Island, NY). Fetal bovine serum was purchased from Biocell Laboratories (Rancho Dominguez, CA) following an extensive testing and selection protocol based on cell attachment, morphology, mitotic expansion without differentiation, and retention of multilineage developmental potential in response to appropriate environmental cues [Lennon et al., 1996]. L-ascorbic acid-2-phosphate (AsAP) was purchased from Wako Chemical (Osaka, Japan), 1, 25-$(OH)_2$ vitamin D_3 (vitamin D_3) from Biomol (Plymouth Meeting, MA), and TRI Reagent from Molecular Research Center (Cincinnati, OH). Radioisotopes were obtained from Amersham International (Arlington Heights, IL). All other routine reagents used were of analytical grade.

Cell Preparation and Culture Methods

Fresh bone marrow (10 ml) was obtained by routine iliac crest aspiration from normal human donors (ages 10 to 58 years) after informed consent. MSCs were isolated from these marrow aspirates using methods modified from those described previously [Haynesworth et al., 1992a]. Briefly, 10 ml of marrow was added to 20 ml of DMEM containing 10% fetal bovine serum from selected lots (Control Medium), and centrifuged to pellet the cells and remove the fat layer. Cell pellets were then resuspended and fractionated on a density gradient generated by centifuging a 70% Percoll solution at 13,000*g* for 20 min. The MSC-enriched low density fraction was collected, rinsed with Control Medium, and plated at 1×10^7 nucleated cells/60 cm^2 dish. The MSCs were cultured in Control Medium at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. When culture dishes became near-confluent, cells were detached with 0.25% trypsin containing 1mM EDTA for 5 min at 37°C, and subsequently replated at 5×10^3 cells/cm^2 for continued passaging. For in vitro osteogenic assays, MSCs were replated in Control Medium at 3×10^3 cells/cm^2 in 6-well (10 cm^2) tissue culture plates. The following day (Day 0), fresh Control Medium was provided, and the cells were subsequently grown in the absence or presence of Osteogenic Supplements (OS) (100 nM Dex, 10 mM βGP and 0.05 mM AsAP). Base media for comparison included DMEM, α-MEM, BGJ_b and DMEM/Hams F-12 (1:1). In some experiments, Dex was used at concentrations ranging from 1 to 1000 nM, βGP was used at concentrations ranging from 1 to 10 mM, and AsAP was used at concentrations ranging from 0.01 to 4 mM. Freshly prepared 0.25 mM AsA was also compared against AsAP. Media changes were performed twice weekly, and the media volume was 2 ml per dish unless otherwise specified. At days 4, 8, 12, and 16 cultures were assayed as described below.

Cell Proliferation Assay

Cell proliferation was measured in triplicate cultures using a modification [Lennon et al., 1995] of the crystal violet dye-binding method [Westergren-Thorsson et al., 1991]. Cultures were rinsed twice with Tyrodes balanced salt solution, fixed with 1% gluteraldehyde (v/v) in Tyrode's for 15 min, rinsed twice with deionized water, and air-dried. Cultures were then stained with 0.1% crystal violet (w/v) in water for 30 min. After washing, crystal violet dye was extracted from the cells by 4 h rotary incubation at 25°C with 1% Triton X-100 (v/v in water). Absorbance of the resulting Triton extract was read at 595 nm on a microplate reader (Bio-Rad). Absorbance values were converted into absolute cell numbers based on established standard curves.

Alkaline Phosphatase Assay

Alkaline Phosphatase (APase) enzyme activity of the cell layer was measured in triplicate cultures by rinsing twice with Tyrode's balanced salt solution, and then incubating the cells with 5 mM p-nitrophenyl phosphate in 50 mM glycine, 1 mM $MgCl_2$, pH 10.5, at 37°C for 5 to 20 min. Alkaline phosphatase enzyme activity was calculated after measuring the absorbance of p-nitrophenol product formed at 405 nm on a microplate reader (Bio-Rad). Enzyme activity was expressed both as nmol of p-nitrophenol/min, and p-nitrophenol/min/10^6 cells.

Histochemical Analyses

Alkaline phosphatase histochemistry was performed for 1 h at 25°C as recomended by the manufacturers instructions contained in Sigma Kit #85. During incubation, culture dishes were

protected from drying and direct light. Dishes were rinsed with deionized water, and air-dried prior to viewing. Selected specimens were subsequently stained for mineral by the von Kossa method. Cell layers were fixed with 10% formalin for 1 h, incubated with 2% silver nitrate solution (w/v) for 10 min in the dark, washed thoroughly with deionized water, and then exposed to bright light for 15 min.

Northern Blot Analysis of Osteocalcin mRNA

Human MSC cultures were grown in Control and OS Medium for 14 days. Control and OS treated cells were then cultured in Control Medium, or Control Medium containing βGP and AsAP respectively, with and without 10 nM vitamin D_3 for 48 h. On day 16, total RNA was extracted with TRI-Reagent according to the manufacturers instructions. In brief, after washing cells with sterile DMEM, RNA was extracted with TRI-Reagent/chloroform solution and precipitated with isopropanol. RNA was dissolved in RNase-free DEPC-treated water, and quantitated by measuring absorbance at 260 nm. Aliquots (20 µg) of total RNA were separated through a formaldehyde-containing 0.9% agarose gel, and then transferred to a nitrocellulose membrane using capillary action [Thomas, 1980]. RNA was cross-linked to the membrane using ultraviolet radiation (Stratagene, La Jolla, CA) and the membrane was incubated for 8 h at 42°C in a solution containing 6 X SSC (1 X SSC = 0.15 M NaCl and 0.015 M sodium citrate), 50% formamide, 7.5 X Denhardt's Solution (1 X = 0.1% BSA, 0.1% Ficoll, and 0.1% polyvinyl pyrolidone), and 0.1 mg/ml heat-denatured salmon sperm DNA. The membrane was hybridized to a cDNA encoding human osteocalcin, which was generated by RT-PCR of human bone RNA [Thiede et al., 1994]. This cDNA was labeled with dCTP using random priming, and placed into fresh prehybridization solution at 1.5×10^6 CPM/ml. Following hybridization at 42°C for 18 h, filters were first washed at room temperature in a solution containing 1.0 X SSC/0.1% SDS and then at 55°C in 0.3 X SSC/0.1% SDS. Washed blots were then exposed to Kodak X-AR5 X-ray film at -70°C with intensifying screens for up to 2 days. Ethidium bromide staining of 18S and 28S rRNA was used to demonstrate similar loading of samples.

Calcium Assay

Cell layers were rinsed twice with PBS and scraped off the dish in 0.5 N HCl. The cell layers were extracted by shaking for 4 h at 4°C, then centrifuged at 1000*g* for 5 min, and the supernatant was used for calcium determination according to the manufacturer's instructions contained in Sigma Kit #587. Absorbance of samples was read at 575 nm 3 min after the addition of reagents. Total calcium was calculated from standard solutions prepared in parallel, and expressed as µg/dish.

X-Ray Diffraction Analysis

To analyze the mineral deposited as a result of OS treatment, MSCs were grown for 16 to 20 days in the presence of 100 nM Dex, 0.05 mM AsAP, and 10 mM βGP. The cell layers were washed twice with PBS, scraped from the surface of the dish, pelleted by brief centrifugation, deposited as a thin layer on a flat quartz crystal, air dried, and fixed with 10% neutral buffered formalin. Additional specimens were generated by growing MSCs directly on glass coverslips, rinsing twice with PBS, and fixing with 10% neutral buffered formalin. Unedited powder diffraction patterns of the dried cell layers were generated using a copper X-ray tube at a 6° take-off-angle, 1° divergence slit, graphite diffracted beam monochromator, and scintillation detector. Intensities (X-ray counts) were taken at 0.02°2Θ increments.

Statistics

Statistical analyses were performed using Students one-tailed *t*-test.

RESULTS

Cultivation and Passaging of Human Mesenchymal Stem Cell Cultures

When the MSC-enriched fraction of mononuclear cells from the Percoll gradient was plated in medium containing our selected lot of fetal bovine serum, approximately one MSC colony developed per 10^5 nucleated cells placed in culture. The remaining cell population was removed from the dish during medium changes and subsequent passaging. The adherent MSCs gave rise to colonies which first became visible around day 5 of culture as cells exhibited their characteristic spindle-shaped morphology. Colony size grew quickly between days 6 and

12, and the monolayer of MSC colonies were passaged when the cell density approached 80–90% confluence as previously described [Haynesworth et al., 1992a,b]. Subcultured MSCs replated at 30% confluence in new dishes attached uniformly throughout the culture plates. Typically, 80–90% confluence was reached by day 8 for most of the passaged cells, at which time, MSCs were subcultured again and used for osteogenic assays. Retention of the MSC phenotype following subculture has been described previously, and is based on cell surface immunostaining by MSC-specific monoclonal antibodies [Haynesworth et al., 1992a]. In general, the results presented here reflect experiments performed with MSCs derived from either 2nd or 3rd passage, although similar results were obtained with MSCs derived from samples which were extensively subcultured or cryopreserved [Bruder et al., 1997].

Induction of Alkaline Phosphatase Activity and Proliferation by OS in Human MSC Cultures

During the 16 day assay period, MSCs cultured with optimized OS (containing 100 nM Dex, 10 mM βGP, and 0.05 mM AsAP) underwent a dramatic change in cellular morphology which was accompanied by a significant increase in APase activity. During the latter half of this culture period, the deposition of a calcified matrix on the surface of the culture dish became evident by von Kossa staining, calcium quantitation, and X-ray diffraction. This difference in APase activity and mineral deposition in cultures grown with and without OS was grossly apparent, occurred for all marrow donors, and is illustrated by one representative sample in Figure 1. MSCs cultured with OS showed a change in their morphology from spindle-shape to cuboidal in as little as 2 days, and was more apparent by 4 days (Fig. 2a,e). On day 4, approximately 30–40% of cells were APase-positive when cultured in OS Medium, whereas control cultures contained APase-positive cells at a much lower frequency. The number of total cells was also greater in OS cultures throughout the period of study. Additionally, the APase-positive cells in control cultures were always spindle-shape with only rare APase-positive cuboidal cells. By day 8, nearly all cells in OS cultures were cuboidal or polygonal, and over 80% were APase-positive. While control cultures grew as a uniform sheet of cells, OS cultures began to form multilayered

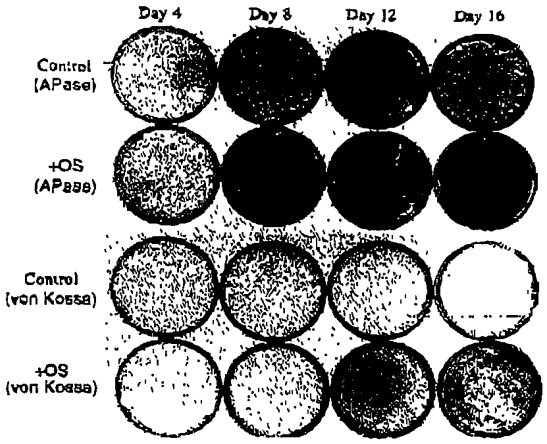


Fig. 1. Effect of osteogenic supplements (OS) on APase and von Kossa staining of human MSC cultures. MSCs were seeded at 3×10^1 cells/cm^2 in 35 mm dishes, grown in Control Medium or medium containing OS for the indicated times, and fixed and stained for APase or mineral deposition by the von Kossa technique as described in Materials and Methods. (Magnification = 0.38 X.)

nodular structures as the apparent result of coalescing cellular aggregates (Fig. 2b,f). At day 12, OS cultures contained a well-developed uniform sheet of bone-like material throughout the entire dish. The formation of these nodular aggregates appeared to expose bare tissue culture plastic in the internodular regions (Fig. 2g,h). Early regions of mineralization were evident in day 12 OS cultures, but never in control cultures which grew in a whirling pattern on the dish (Fig. 2c,g). By day 16, extensive mineralization occurred throughout OS cultures, but was never detected in control cultures. Although the multilayered high density day 16 control samples modestly increased their number of APase-positive cells, the cellular morphology always remained spindle-shaped and did not appear like MSCs grown in OS Medium (Fig. 2d,h).

In an effort to study the effect of Dex on MSCs, with particular emphasis on osteogenic lineage induction, cultures were incubated with different doses of Dex from 1 to 1000 nM for up to 16 days. Cell morphology, proliferation, APase activity, and mineralization were assayed on days 4, 8, and 16. Figure 3a demonstrates that on day 8, MSCs cultured in OS containing 1 to 1000 nM Dex increase APase activity per cell 3- to 8-fold compared to cells grown in Control

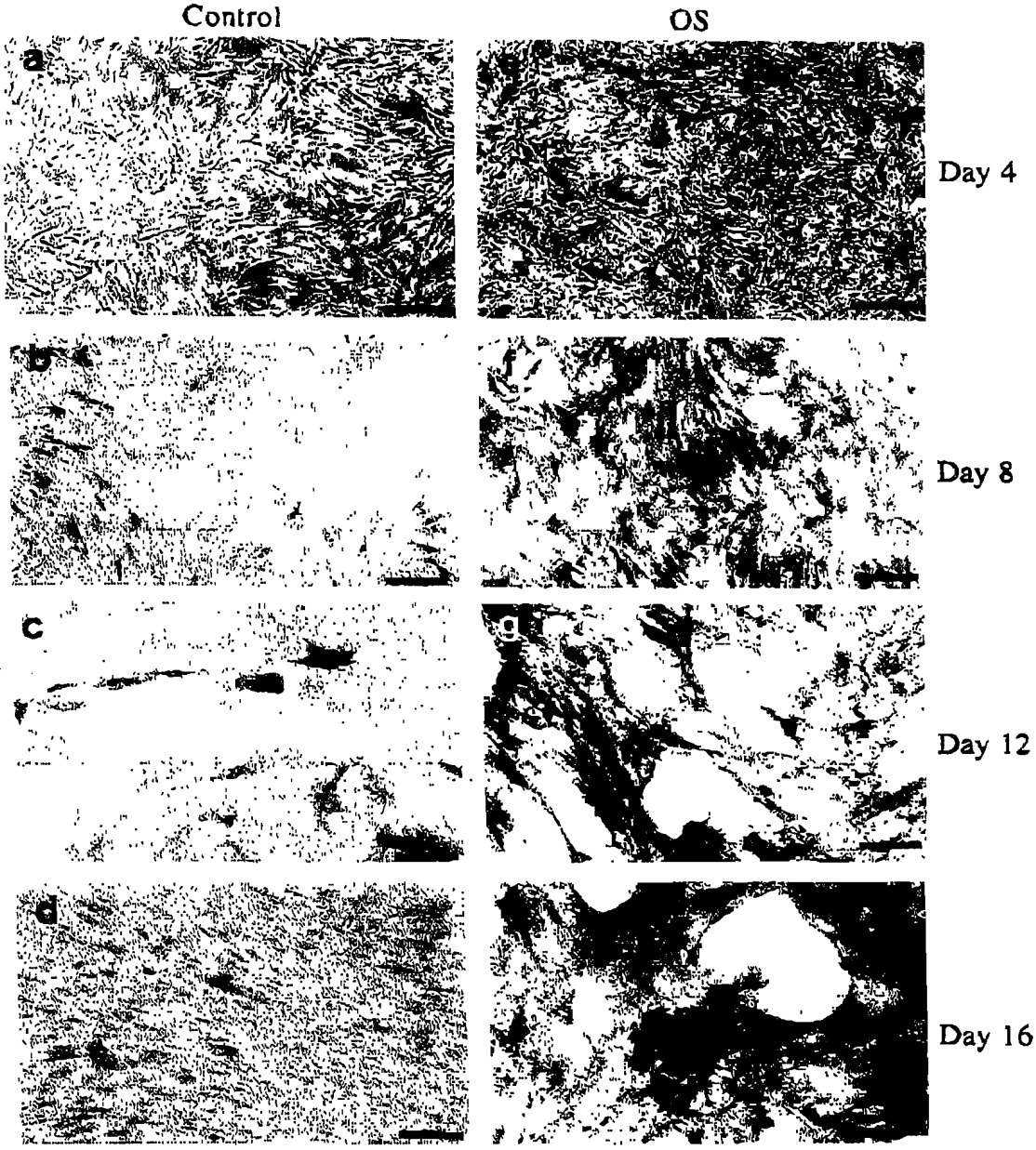
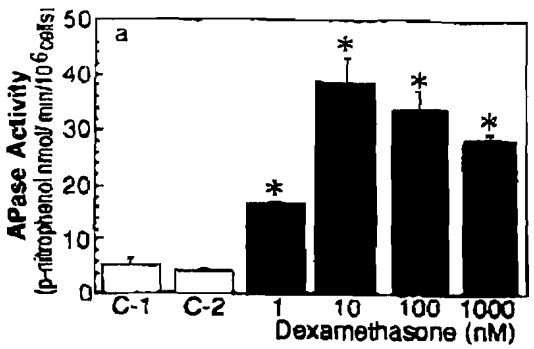


Fig. 2. Effect of OS on cell morphology, APase expression, and mineral deposition in human MSC cultures. All specimens were stained by APase and von Kossa histochemical techniques as described in Materials and Methods. Phase contrast microscopy demonstrates the spindle-shaped morphology of control MSCs at day 4 (a), while cells grown with OS have become polygonal, more numerous, and APase-positive (e). By day 8, control cells are observed as nearly confluent whorls (b), while OS-treated cells have begun to form nodular aggregates which are strongly stained for APase (black and gray)(f). At day 12, spindle-shaped APase-positive cells are present in control cultures (c), but cells grown with OS have formed nodular aggregates and begun to mineralize their matrix (shown as black granular deposits) (g). Control cultures are uniformly dense by day 16 (d), while OS-treated cells have deposited abundant von Kossa staining mineral (h). The formation of nodular aggregates in OS-treated cells results in the exposure of bare tissue culture plastic visible in (g) and (h). Scale bar = 500 μm in a,b,e,f and 200 μm in c,d,g,h.



Medium alone, or cells grown in Control Medium with βGP and AsAP added. At 1 nM, Dex significantly stimulated the APase activity, however, cells appeared fibroblastic with a spindle-shape morphology and no mineralized nodule formation occurred (Fig. 3b). Maximum APase activity per cell was observed in cultures treated with OS containing 10 nM Dex, although osteoblastic morphology and mineral deposition were substantially greater at 100 nM Dex (Fig. 3c,d). Cultures treated with OS containing 1000 nM Dex demonstrated more mineralization than those containing 100 nM Dex, however, by day 16 the cell layers cultured with 1000 nM Dex usually detached from the dish (Fig. 3e). There was an increase in cell number in cultures exposed to 1 to 1000 nM Dex, with the maximal effect observed at 1 nM (data not shown). Although human MSCs can differentiate into cartilage or fat, under the tissue culture conditions used in this study, neither chondrogenic nor adipogenic differentiation were ever observed at any Dex dose as determined by Toluidine Blue or oil red O staining, respectively.

Since ascorbic acid (AsA) functions as a cofactor in the hydroxylation of proline and lysine residues in collagen [Schwartz, 1985; Schwartz et al., 1987; Aronow et al., 1990], as well as increasing the synthesis of non-collagenous bone matrix proteins [Graves et al., 1994], its daily addition to osteogenic cell cultures is now considered essential. However, because of its instability in solution at 37°C and neutral pH, a

Fig. 3. a: Dose dependent effect of dexamethasone (Dex) on APase activity in 8 day human MSC cultures. Control samples were grown in either Control Medium (C-1) or Control Medium + 0.05 mM AsAP + 10 mM βGP (C-2). Various concentrations of Dex were added to Control Medium (C-2). Samples were harvested on day 8, and APase activity and cell number were determined as described in Materials and Methods. The results represent the mean ± SD of triplicate cultures of one representative experiment. *$P < 0.05$; (compared to the C-1 control value). Light micrographs of human MSC cultures grown with different doses of Dex and stained for APase and mineral on day 16. Cultures treated with 1 nM Dex (b) showed a fibroblastic spindle-shape morphology with no mineralized nodule formation, but those exposed to 10 nM Dex (c) are intensely stained for APase. Note the presence of few cells with polygonal osteoblastic morphology and mineral deposition, whereas 100 nM Dex treated cultures (d) showed more mineralizing APase-positive structures. Cultures treated with 1000 nM Dex (e) possessed maximum mineralization, but most of the cells eventually detached from the plate, resulting in a bare tissue culture dish. Scale bar = 220 μm.

stable analogue, AsAP, has been developed which has similar activity in tissue culture [Hata and Senoo, 1989]. We have investigated the effect of various concentrations of AsAP (0.05 to 4.0 mM) on in vitro osteogenesis of MSCs in order to eliminate the need for daily supplementation of fresh AsA (0.25 mM, or 50 μg/ml). AsAP concentrations of 1 to 4 mM had a toxic effect by 4 days of culture as noted by the presence of dead floating cells and the deposition of large birefringent crystals. The maximal increase in APase activity, cell proliferation and mineralized matrix production was obtained with OS containing 0.05 mM AsAP (Table I). The effect of this dose was nearly identical to that of the historical gold-standard, fresh 0.25 mM AsA added daily. The addition of AsAP to MSCs in the absence of Dex and βGP did not cause a significant change in APase activity, although the cell number increased independent of dose.

Figure 4 illustrates the effect of optimized OS Medium (100 nM Dex, 10 mM βGP, and 0.05 mM AsAP) on APase expression and cell proliferation during osteogenic differentiation of human MSCs in vitro. APase activity in OS cultures is elevated by day 4, and continues to climb until peaking at day 12 (Fig. 4a). APase activity/min/10^6 cells as well as total APase activity/min declined between days 12 and 16. This drop in APase activity in MSC cultures on day 16 is reproducible, and correlated with increasing mineral deposition in nearly every culture studied. Cell number was also significantly greater in OS treated cultures than in control cultures at day 8 and beyond (Fig. 4b). Finally, cell proliferation in OS cultures supplemented with AsAP was identical to those fed AsA daily through day 12, and actually, AsAP supported greater proliferation by day 16 (data not shown). Additional evidence for the stability and utility of AsAP is found in the observation that analogous biological results can be observed by using a molar amount of AsAP only one-fifth that of AsA (0.05 mM vs. 0.25 mM) (Table I).

TABLE I. Effect Ascorbic Acid-2-phosphate Concentration on APase Activity and Cell Proliferation in Human MSC Cultures on Day 8

	APase activity (p-nitrophenol nmol/min)	Cell number (×1000)
Control Medium	0.56 ± 0.03	23.20 ± 2.17
Control Medium + βGP + Dex	3.43 ± 0.40*	68.77 ± 1.52*
Control Medium + βGP + Dex +		
AsA (0.25 mM)	5.41 ± 0.16*†	77.86 ± 0.78*†
AsAP (0.05 mM)	5.07 ± 0.59*†	73.49 ± 1.37*†
AsAP (0.10 mM)	3.93 ± 0.48*	65.60 ± 0.73*†
AsAP (0.25 mM)	3.86 ± 0.14*	61.53 ± 2.55*†
AsAP (0.50 mM)	4.05 ± 0.22*	59.18 ± 2.24*†

Effect of ascorbic acid-2-phosphate (AsAP) concentration on APase activity and cell proliferation in human MSC cultures. Cells were initially plated in Control Medium, and the following day the medium was replaced with either fresh Control Medium, Control Medium containing 10 mm βGP and 100 nm Dex, or Control Medium containing 10 mm βGP, 100 nm Dex, and various concentrations of AsAP or fresh ascorbic acid (AsA). Samples were harvested on day 8, and APase activity and cell number were determined as described in Materials and Methods. The results represent the mean ± SD of triplicate cultures of one representative experiment. *$P < 0.0005$ (compared to Control Medium); †$P < 0.05$ (compared to Control Medium containing βGP and Dex only).

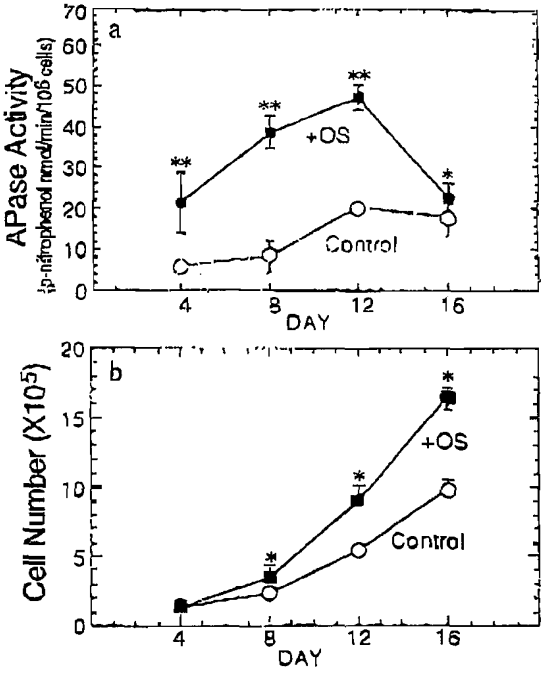


Fig. 4. APase activity and cell proliferation in human MSC cultures grown in Control or OS Medium on days 4, 8, 12, and 16. Samples were harvested at the indicated days, and APase activity and cell number were determined as described in Materials and Methods. a: APase activity/min/10^6 cells; b: cell number (×10^5). The results represent the mean ± SD of triplicate cultures of one representative experiment. *$P < 0.05$; **$P < 0.01$ (compared to the control value).

Although the timing of the peak APase activity of different donors varied from days 8 to 12, the phenomenon of osteogenic differentiation manifest by APase induction prior to mineral accumulation between days 12 and 16 was observed in cultures from every single donor. In order to demonstrate ubiquity of the osteoinductive phenomenon while simultaneously presenting the donor variability in absolute values of APase activity, Table II lists the APase activities at day 8 for 7 different donors' MSC cultures grown with or without OS. These data indicate that day 8 APase activity per cell increases 1.5- to 6.4-fold depending on the donor. For emphasis, it must be noted that some of these donors did not exhibit peak APase activity until day 12. There was no correlation between the age of the donor and the fold increase in APase activity of MSCs on day 8, nor was there a correlation between the basal level of APase activity in control cultures and the fold stimulation in response to OS. A similar variability in APase activity by osteoblast-like cells derived from bone marrow of different donors has also been reported [Kassem et al., 1991; Cheng et al., 1994].

Human MSCs Mineralize their Extracellular Matrix in Response to OS

Human MSCs were also studied for their ability to mineralize the extracellular matrix

TABLE II. Effect of OS on APase Activity in Human MSC Cultures from Various Patients on Day 8

Patient (age)	APase activity (p-nitrophenol nmol/min/10^6 cells) Control	+OS	Fold stimulation
1 (10)	45.30 ± 3.30	75.17 ± 2.95*	1.65
2 (32)	9.26 ± 0.99	32.67 ± 0.15**	3.52
3 (32)	3.01 ± 2.50	7.59 ± 1.60**	2.52
4 (37)	9.98 ± 3.26	36.30 ± 6.50**	3.65
5 (39)	42.03 ± 1.91	65.85 ± 2.10**	1.56
6 (47)	10.13 ± 1.01	16.81 ± 1.83*	1.56
7 (58)	5.36 ± 1.10	34.39 ± 2.70**	6.41

Effect of OS on APase activity in human MSC cultures from various patients on day 8. MSCs from multiple patients were grown in the absence or presence of OS, harvested at day 8, and APase activity and cell number were determined as described in Materials and Methods. The results represent the mean ± SD of triplicate cultures of one representative experiment from each donor. *$P < 0.01$; **$P < 0.0005$ (compared to the control value).

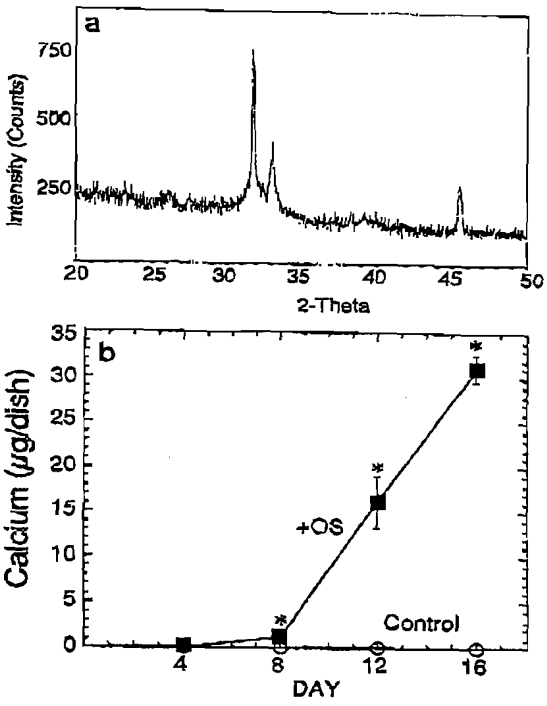


Fig. 5. Mineral deposition in human MSC cultures grown in Control or OS Medium. a: Unedited X-ray powder diffraction pattern of human MSCs grown in OS Medium for 20 days, pelleted onto a quartz crystal, and analyzed as described in Materials and Methods. b: The amount of calcium deposition on days 4, 8, 12, and 16. The cell layers were harvested, and calcium content was determined as described in Materials and Methods. The results represent the mean ± SD of triplicate cultures of one representative experiment. *$P < 0.001$ (compared to the control value).

which they produce when cultured in the presence of OS. Cells grown in Control Medium alone, or Control Medium with βGP and AsAP did not deposit detectable calcium throughout the culture period as measured by von Kossa staining or the sensitive colorimetric quantitative calcium assay. By contrast, MSCs grown with Dex, 10 mM βGP and AsAP deposit a heavily mineralized matrix between days 12 and 16 (Fig. 2g,h). Importantly, this mineralization pattern was distributed throughout the culture dish rather than localized to a few discrete foci like that observed in stroma-derived cell cultures from rat. The X-ray diffraction pattern of the mineral formed in these cultures is remarkable for intensity peaks between 30 and 35° 2Θ (Fig. 5a), the region which is characteristic of bone apatite [Handschin and Stern, 1992]. These peaks are also coincident with the

position of peaks for the reference standard (JCPDS 9-432) of fully crystalline hydroxyapatite. The mineralization induced in response to OS does not appear to be the result of dystrophic calcification due to cell death or necrosis. Likewise, the absence of mineral in cultures grown in Control Medium with βGP and AsAP, or even OS cultures maintained in α-MEM or DMEM/F-12, further refute the possibility that 10 mM βGP is a supersaturating dose which leads to nonphysiologic spontaneous crystal deposition. Figure 5b graphically illustrates that no calcium was detected at any time in control cultures, but cultures treated with OS showed a significant increase in calcium content of the cell layer as early as day 8, with more substantial accumulation occurring by days 12 and 16. Here again, the absolute values of calcium deposition ranged from patient to patient, but the presence of the phenomenon did not vary (Figs. 5,8,9c).

Human MSCs Express Osteocalcin mRNA in Response to 1, 25-$(OH)_2$ Vitamin D_3

Northern blot analysis was performed on total cellular RNA extracted from human MSCs grown in the absence or presence of OS, with and without a 48 h exposure to vitamin D_3. Figure 6 illustrates that in cells cultured in either Control Medium or OS Medium without vitamin D_3, osteocalcin mRNA was undetectable. In the presence of vitamin D_3, however, osteocalcin mRNA was induced in both cultures grown in Control Medium and OS Medium. Importantly, those cultures grown in Control Medium with vitamin D_3, which were never stimulated into the osteogenic lineage by Dex, expressed considerably more osteocalcin mRNA than those cultures which were grown in the presence of OS for 14 days prior to their vitamin D_3 exposure. This observation is consistent with others [Beresford et al., 1984; Cheng et al., 1994] who have demonstrated attenuation of vitamin D_3 responsiveness when overtly osteoblastic cells are exposed to Dex.

Basal Tissue Culture Medium Influences Expression of the Osteogenic Potential of Human MSCs

Different nutrient-containing basal media were found to influence the osteogenic differentiation of human MSCs. Cultures were plated in Control Medium (containing DMEM) and then switched to various base media 1 day after

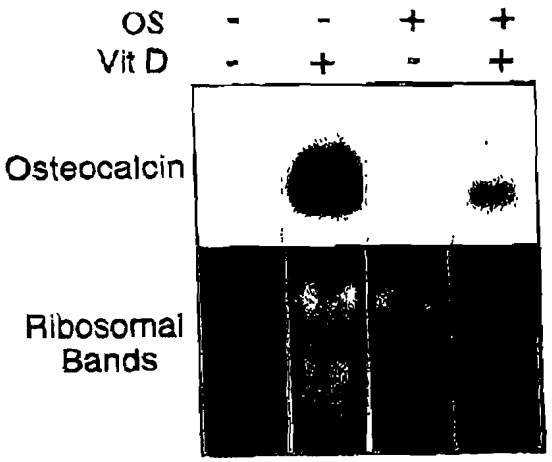


Fig. 6. Expression of osteocalcin mRNA in human MSC cultures grown in Control or OS Medium in the absence or presence of vitamin D_3. Cultures were grown in either Control or OS medium for 14 days, and then exposed to Control Medium, or Control Medium containing βGP and AsAP, respectively, with and without 10 nM vitamin D_3 for the final 40 h of culture. **Upper panel:** Northern blot for osteocalcin mRNA in Control and OS-treated cultures grown in the absence and presence of vitamin D_3. **Lower panel:** Ethidium bromide staining of the agarose gel for ribosomal RNA demonstrates similar loading in each lane.

initial plating (Day 0). For these experiments, all media contained the same selected lot of fetal bovine serum at 10% (v/v). As shown in Figure 7, specimens grown in BGJ_b + OS died and detached from the plate by day 12. Cells grown in DMEM/F-12 + OS showed only a modest increase in APase activity throughout the culture period. By contrast, cultures grown in DMEM or α-MEM increased their population of APase-positive cuboidal cells substantially by day 8 when grown in the presence of OS. It is critical to note that the background level of APase staining in α-MEM cultures is quite higher than that observed in DMEM cultures. Quantitative assessment of APase demonstrated maximal activity in α-MEM + OS throughout the entire culture period, but basal APase expression in these control cultures was relatively high, and not substantially different than DMEM+OS (Table III). DMEM alone showed the lowest basal APase activity compared to any other medium tested. Although α-MEM+OS resulted in the maximum absolute APase activity, the fold stimulation over the respective control (medium without OS) was

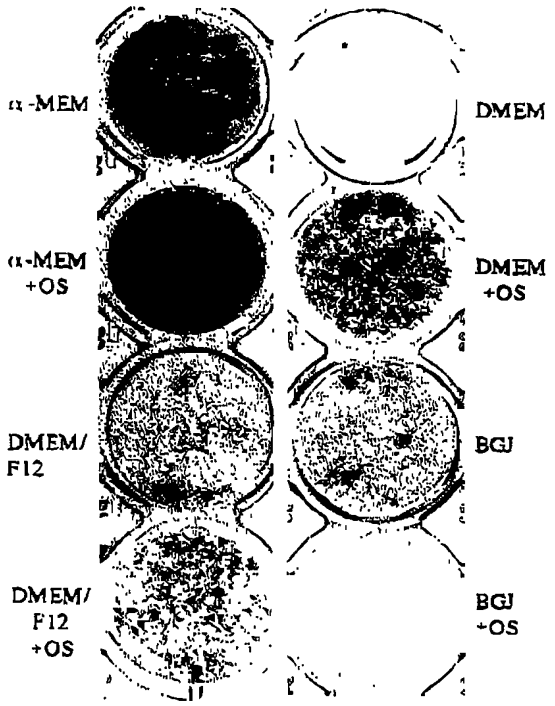


Fig. 7. APase staining of human MSC cultures grown in the absence or presence of OS in various base media on day 12. Cells were initially plated in DMEM Control Medium, and the following day the medium was changed to that indicated in the figure. On day 12, triplicate samples were stained for APase as described in Materials and Methods. Note that cells grown in BGJ_b + OS detached from the plate. (Magnification = 2.2 X.)

greater in samples cultured in DMEM+OS. Specifically, addition of OS to DMEM increased the APase activity by about 13-, 11-, and 6-fold on days 8, 12, and 16 respectively, while the addition of OS to α-MEM increased APase activity only 4-, 4-, and 2-fold, for the same culture periods. The addition of OS to DMEM significantly stimulated the proliferation of MSCs during the entire culture period, but inhibited proliferation of cells cultured in α-MEM (Table III). As compared to DMEM and α-MEM, MSCs grown in DMEM/F-12 in both the absence and presence of OS showed a low cell number (data not shown). Interestingly, although α-MEM + OS cultures exhibited maximal APase activity, mineralization was sparsely detected by von Kossa staining. Cultures grown in DMEM + OS contained less total APase activity, but were substantially more mineralized than those grown in α-MEM + OS.

Effect of Initial Seeding Density on Alkaline Phosphatase Induction, Cell Proliferation and Mineralization

Human MSCs grown in OS Medium with an initial cell plating density of either 3 x 10^3/cm² or 5 x 10^3/cm² showed no significant difference in APase activity beyond day 8, and no significant difference in cell number after day 4 (data not shown). However, mineralized matrix deposition by von Kossa staining and quantitative calcium determinations were positively correlated with the higher initial plating density. No calcium was detected in cultures grown in Control Medium at any seeding density up to day 16. Dishes seeded with 5 x 10^3 cells/cm² produced significantly more calcium deposits compared to those seeded at 3 x 10^3 cells/cm² from day 8 onward (Fig. 8). Cells plated at 5 x 10^3/cm² showed increases of 8%, 101%, and 37% in calcium content on days 8, 12, and 16, respectively, when compared to identical cells grown after an initial plating density of 3 x 10^3/cm². Culture dishes seeded with 7.5 x 10^3 cells/cm² deposited even more mineral than cultures seeded at 5 x 10^3/cm², however, the greater seeding density typically resulted in detachment of the cell layers between days 12 and 16.

Effect of Tissue Culture Medium Volume per Dish on Alkaline Phosphatase Induction, Cell Proliferation, and Mineralization

We also investigated the effect of growing human MSCs in 10 cm² dishes containing 1 vs. 2 ml of culture media per dish. For all previous experiments within this report, cultures were routinely fed 2 ml of medium. Interestingly, while there was no recognizable difference in morphology, APase activity, or cell proliferation at any time in cultures grown with 1 or 2 ml of either Control Medium or OS Medium (Table IV), there was a significant increase in mineralization of cultures grown in 1 ml of medium (Fig. 9c). Despite the dramatic increase in cell surface APase activity of cultures grown with OS, measurements of soluble APase activity in the media collected from cells grown either under Control or OS conditions are extremely low and not statistically different. Furthermore, there was no difference in soluble APase activity in the media from cultures grown with 1 or 2 ml of either Control or OS medium, thus refuting the possibility that advanced mineralization in cultures fed 1 ml is simply a conse-

TABLE III. Effect of Basal Tissue Culture Medium on APase Activity and Cell Proliferation in Human MSC Cultures

	APase activity (p-nitrophenol nmol/min/10^6 cells)				Cell number (×1000)			
Day	DMEM	DMEM + OS	α-MEM	α-MEM + OS	DMEM	DMEM + OS	α-MEM	α-MEM + OS
4	0.52 ± 0.33	4.63 ± 0.41*	0.45 ± 0.28	3.67 ± 0.31*	6.45 ± 1.20	9.14 ± 0.62*	11.92 ± 0.99	10.79 ± 0.78
8	0.74 ± 0.26	9.31 ± 2.15*	1.18 ± 0.16	18.08 ± 3.95*	27.67 ± 2.76	46.69 ± 9.32*	80.69 ± 0.58	68.13 ± 3.96*
12	1.59 ± 0.60	17.11 ± 3.65*	10.53 ± 1.96	38.13 ± 4.77*	72.40 ± 5.86	92.49 ± 10.05*	122.00 ± 6.41	97.49 ± 2.33*
16	6.28 ± 1.34	39.17 ± 7.26*	33.10 ± 1.40	80.34 ± 6.17*	87.19 ± 6.26	129.08 ± 9.04*	171.97 ± 21.59	141.72 ± 6.07*

Effect of basal tissue culture medium volume on APase activity and cell proliferation in human MSC cultures. Cells were initially plated in DMEM Control Medium in 24-well plates, and the following day the medium was changed to DMEM or α-MEM with and without OS. Samples were harvested on days 4, 8, 12, and 16, and APase activity and cell number were determined as described in Materials and Methods. The results represent the mean ± SD of triplicate cultures of one representative experiment. *$P < 0.05$ (compared to the corresponding control cultures without OS).

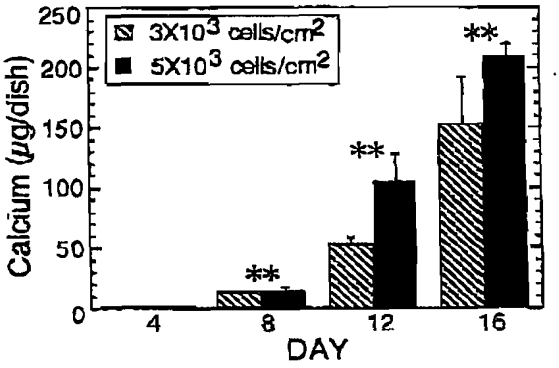


Fig. 8. Effect of initial plating density on calcium deposition by human MSCs grown in the presence of OS on days 4, 8, 12, and 16. Cells were initially seeded at 3 × 10^3/cm² or 5 × 10^3/cm², switched to OS Medium the following day, and harvested at the indicated times. Calcium deposition was determined as described in Materials and Methods. The results represent the mean ± SD of triplicate cultures of one representative experiment. No significant difference in cell number was detected beyond day 4. **$P < 0.01$ (compared to dishes seeded at 3 × 10^3/cm²).

TABLE IV. Effect of Cell Culture Medium Volume on APase Activity and Cell Proliferation in Human MSC Cultures on Day 16

	Control medium	+OS medium
APase Activity (p-nitrophenol nmol/min/10^6 cells)		
1 ml medium	76.53 ± 4.63	100.91 ± 27.54
2 ml medium	76.50 ± 1.81	121.27 ± 10.89
Cell Number (×1000)		
1 ml medium	227 ± 6	537 ± 54
2 ml medium	230 ± 4	561 ± 123

Effect of cell culture medium volume on APase activity and cell proliferation in human MSC cultures grown in the absence or presence of OS on day 16. Cells were initially plated in 2 ml of Control Medium. The following day, the medium was changed and samples were grown in dishes containing 1 or 2 ml of Control or OS Medium which was then changed twice weekly. APase activity and cell number were determined on days 4, 8, 12 and 16 as described in Materials and Methods. The results represent the mean ± SD of triplicate cultures of one representative experiment. All P values > 0.3 (comparing samples fed 1 or 2 ml for each assay, both with and without OS).

quence of increased soluble APase concentration. The photomicrographs in Figure 9a and b illustrate that cell morphology is unchanged by reducing the volume of medium to 1 ml, although the extent of mineralization is dramatically increased.

DISCUSSION

This study demonstrates that purified, culture-expanded human MSCs can be directed into the osteogenic lineage in vitro, culminating in mineralized matrix production, and thereby establishing a system for studying human osteoblast differentiation from postnatal stem cells. The addition of Osteogenic Supplements (100 nM Dex, 10 mM βGP, and 0.05 mM AsAP) to MSC cultures was capable of inducing rapid osteogenesis as defined by the appearance of osteoblastic cell morphology, increased expression of APase, reactivity with anti-osteogenic cell surface monoclonal antibodies [Bruder et al., 1995], the formation of a mineralized extracellular matrix containing hydroxyapatite, and attenuation of vitamin D_3-responsive osteocalcin mRNA synthesis. Physiologic concentrations of glucocorticoid were required for this phenomenon, which was further supported by an ascorbic acid analogue and β-glycerophosphate. This culture system is responsive to

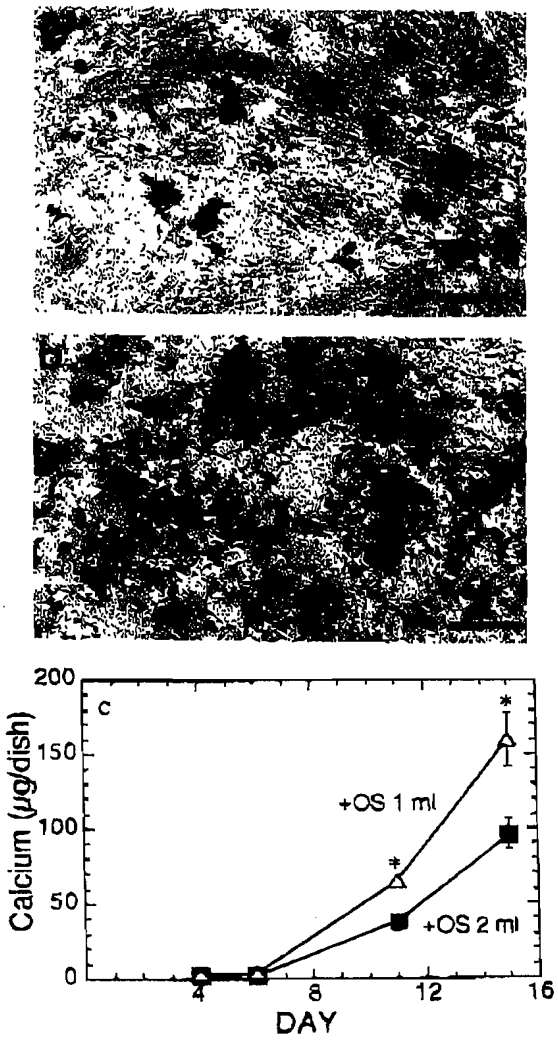


Fig. 9. Phase contrast micrograph of MSC cultures in 10 cm² dishes fed 2 ml (a) or 1 ml (b) of OS Medium, and stained for APase and mineral deposits on day 16. Note the increase in mineralization, shown as black deposits, in cultures grown in 1 ml of medium (b) compared to those grown in 2 ml of medium (a). Scale bar = 500 μm. c: Calcium deposition of human MSCs grown in 1 ml or 2 ml of OS Medium on days 4, 8, 12, and 16. The results represent the mean ± SD of triplicate cultures of one representative experiment. No significant difference in cell number or APase activity was observed during the culture period (see Table III). *$P < 0.05$.

subtle manipulations including the basal nutrient medium, dose of physiologic supplements, cell seeding density, and volume of tissue culture medium. With the above in mind, cultured human MSCs provide a useful model for evaluating the multiple factors responsible for the step-wise progression of cells from undifferentiated precursors to secretory osteoblasts, and eventually terminally differentiated osteocytes.

While primary cultures of human MSCs appear morphologically similar to bone marrow stroma-derived cells from human rib [Cheng et al., 1994] or rat long bone [Maniatopolous et al., 1988], the precise conditions for the cultivation of these cell types has a profound effect on cell selection and behavior. The cell seeding density, type of tissue culture plastic, and source of fetal calf serum are known to affect the developmental potential of cultured cells [Maniatopolous et al., 1988; Aronow et al., 1990; LeBoy et al., 1991; Haynesworth et al., 1992a,b; Gallagher et al., 1996]. For these reasons, we have developed a screening protocol which distinguishes serum lots capable of selecting for, and perpetuating, the human MSC phenotype in vitro [Lennon et al., 1996]. Furthermore, unlike other human marrow stroma-derived cell culture systems [Kassem et al., 1991; Cheng et al., 1994; Gundle et al., 1995], we maintain cells in the log phase of growth without allowing them to become confluent and form multilayers, since differentiation of mesenchymal cells is known to be triggered by increasing cell density [Caplan et al., 1983]. This protocol for handling human MSCs supports the retention of stem cell-like properties for many passages, or until such time that the cells are placed in an inductive environment [Haynesworth et al., 1992a; Bruder et al., 1997].

The formation of nodular aggregates by MSCs cultured with OS is strikingly different than that which occurs in bone marrow stroma-derived cell cultures from either rat [Maniatopolous et al., 1988; LeBoy et al., 1991] or chick [Kamalia et al., 1992], as well as those calvarial cell cultures from fetal rat [Bellows et al., 1987; Aronow et al., 1990] or chick [Gerstenfeld et al., 1987]. While these latter systems form spatially discrete nodules upon the dish, with each individual nodule apparently representing one progenitor cell [Bellows and Aubin, 1990], human MSC cultures form a continuously interconnected network of mineralized extracellular matrix. The sheet-like uniformity of this osteogenic response is reminiscent of the intramembranous ossification which occurs in developing calvariae and long bone diaphyses [Caplan and Pechak, 1987]. The observation that virtually all cells are involved in the formation of this bone-like material (Figs. 1, 2, and 3) argues

against the selective induction of a limited number of osteoprogenitors and supports the characterization of this population of cells as homogeneous. The minor variations in regional APase and von Kossa staining likely reflect the extent of differentiation of individual cells and their relative maturity within the osteogenic lineage. While osteoprogenitor cells are generally APase-negative, and those cells which are terminally differentiated, or osteocytic, are also weakly stained for APase, the majority of cells in the midst of lineage progression are highly APase-positive.

The addition of OS to MSC cultures caused a significant increase in APase activity per cell over time, which we interpret to reflect the degree of progression into the osteoblastic lineage. The subsequent decrease in APase activity per cell beyond day 12 correlates with advanced matrix mineralization, modulation of osteocalcin mRNA expression, and terminal osteogenic cell differentiation as MSCs become osteocytes [Bruder and Caplan, 1989, 1990; Nakahara et al., 1990]. Monoclonal antibodies against normal human osteogenic cell surface antigens also react with these cultures, confirming their progression into the bone cell lineage [Bruder et al., 1995]. Interestingly, despite a lower APase activity per cell, cultures grown in DMEM + OS mineralized more extracellular matrix than those grown in α-MEM + OS. This observation could indicate that within the 16 day culture period, DMEM base medium supports further osteogenic differentiation of MSCs than α-MEM. The small amount of mineralization observed in α-MEM + OS cultures, in spite of having higher APase activity than DMEM+OS cultures, could be due to the inhibition of proliferation which occurs in α-MEM + OS, thereby resulting in a sub-threshold density of cells required to signal the mineralization process. This lack of mineralization is consistent with our experiments showing that lower initial cell seeding densities result in less mineral deposition. Another explanation for the small amount of mineralization in α-MEM + OS cultures is that these cells have not been provided with the appropriate microenvironment to complete the lineage translation in vitro by day 16. It is possible that, given more time, α-MEM + OS would foster even more mineralized matrix than DMEM + OS. Although α-MEM medium has been shown to support mineralization of stroma-derived cell cultures in vitro, despite Dex-mediated inhibition of proliferation [Cheng et al., 1994], multiple variables could account for this discrepancy with our data, including differences in the nature and inherent osteogenic potential of marrow-derived cells isolated from different tissues under different conditions. Variations in the base medium favoring maintenance of the MSC phenotype (DMEM), evidenced by MSC-specific immunostaining, or maximal initial recruitment into the osteogenic lineage (α-MEM + OS), noted by the percent APase-positive cells and APase activity, are inherently interesting and warrant further examination.

Paradoxically, glucocorticoids have both stimulatory and inhibitory effects on osteogenic differentiation depending upon the dose, duration, stage of cell differentiation, and species of responding cell. Dex is an absolute requirement for in vitro bone nodule formation and mineralization in rat marrow stroma-derived cell cultures [Maniatopoulos et al., 1988], but it can still influence adipogenesis of these cells in a dose- and time-dependent manner [Beresford et al., 1992]. Rickard et al. [1994] found that treatment of rat marrow stroma-derived cells with Dex results in a population of mature osteoblasts as well as a population of undifferentiated cells which retain the capacity for osteoblastic differentiation with secondary exposure to Dex. Furthermore, Turksen and Aubin [1991] demonstrated that APase-negative osteoprogenitors are dependent upon Dex for differentiation into the bone-forming phenotype, but APase-positive cells did not require Dex to produce bone nodules. Expression of the 1, 25-$(OH)_2$ vitamin D_3-dependent bone matrix protein, osteocalcin, is also influenced by glucocorticoids. Although rat marrow stroma-derived cells upregulate osteocalcin mRNA and protein synthesis in response to vitamin D_3 and Dex [Malaval et al., 1994; Rickard et al., 1994], human osteoblastic cells [Beresford et al., 1984; Wong et al., 1990; Subramanian et al., 1992; Cheng et al., 1994] show a marked reduction in the vitamin D_3-dependent expression of osteocalcin when exposed to Dex. Recent studies by Cheng et al. [1996] further demonstrate that human bone marrow stromal cells decrease their osteocalcin mRNA expression after only one day of Dex exposure, and this Dex-mediated antagonism persists throughout the four week culture period. Our efforts to localize bone Gla protein, or osteocalcin, within the cell layer

by immunohistochemical techniques have met with only limited success, and may reflect the low levels of expression noted by other investigators employing similar in vitro systems [Schepmoes et al., 1991; Cheng et al., 1996]. These studies, combined with the results presented here, suggest that osteocalcin may not be a straightforward or reliable marker for human osteoblastic cell differentiation. The complexity of osteocalcin regulation at the level of transcription and translation is not only dependent on vitamin D_3 and Dex directly [Schepmoes et al., 1991], but the developmental state and species of the cell under investigation. Glucocorticoid-mediated attenuation of vitamin D_3-induced osteocalcin expression in mature osteoblastic cells, reproduced in the current study with human MSC cultures, may reflect a reduction in the number of vitamin D receptors on osteoblastic cells in response to glucocorticoids [Godschalk et al., 1992]. The fact that such attenuation is conserved 48 h after removal of Dex is consistent with other studies demonstrating the sustained effect of Dex on human MSCs up to two weeks following its withdrawal [Bruder and Jaiswal, 1995; Jaiswal and Bruder, 1996].

Since high concentrations of glucocorticoids cause bone loss and decreased osteoblastic activity, as observed in Cushing's Syndrome, the precise pathologic mechanism is at odds with a number of experimental observations. The present study demonstrates that, depending on the tissue culture medium, Dex significantly stimulates MSC proliferation and osteogenic differentiation. One may therefore suggest that in vivo, such bone loss results from stimulating progenitor cell proliferation and differentiation, thus depleting the reserves of available precursor cells. Those investigators who observe inhibition of proliferation in vitro, however, would propose that a reduction in the number of osteoprogenitor cells occurs through an inhibitory pathway. By either mechanism, the end result is a diminution in the available pool of bone-forming cells. In light of the inconsistencies regarding Dex's action on cells of the osteogenic lineage, the role of glucocorticoids in bone physiology will continue to require substantial future efforts.

Our study further shows that while a higher initial plating density did not result in significantly different cell numbers beyond day 4, or different APase activity beyond day 8, there was significantly more mineral deposited in cultures seeded at higher densities. At day 4, differences in cell number under each plating condition simply reflect the difference in initial seeding density (data not shown). The number of cells after day 4 was independent of the initial seeding density over the range of 3×10^3 to 5×10^3 cells/cm^2. In rat marrow stroma-derived cell cultures [Herbertson and Aubin, 1995], like those of chick [Kamalia et al., 1992], nodule formation and especially the onset of mineralization are density-dependent: both occurred earlier when cells were plated at higher densities. Together, these observations suggest that the extent of eventual mineralization is, in part, linked to events which occur upstream in the differentiation process. This hypothesis is consistent with other studies of stem cell differentiation, which demonstrate that committment to an end-stage phenotype is determined at the beginning of the pathway [Shimizu and Bode, 1995]. Additional evidence supporting the role of cell-cell interactions comes from our experiments in which the tissue culture medium volume was reduced from 2 ml to 1 ml. Here again, no difference in cell number or APase activity was observed, although significantly more mineralization occurred in those cultures exposed to less medium. Because osteoblastic cells are known to elaborate soluble factors which regulate their own differentiation [Van Der Plas and Nijweide, 1988; Hughes and McCulloch, 1991], reducing the volume of culture medium would result in an increase in the effective concentration of those soluble factors. Different lots of fetal bovine serum, each with a slightly different complement of bioactive factors, are also known to influence the extent of mineralization [Aronow et al., 1990; Gallagher et al., 1996]. With this in mind, we interpret these data to suggest that differentiating human MSC cultures secrete autocrine or paracrine factors that act locally to stimulate expression of the mature osteoblast phenotype. The elaboration and identification of soluble osteoinductive factors by MSCs undergoing osteogenic differentiation is an area of active investigation [Jaiswal and Bruder, 1997].

The isolation of human MSCs and their cultivation under the conditions described in this report provide a system for analyzing the events of MSC committment and osteogenic differentiation into fully functional secretory osteoblasts and osteocytes. By carefully optimizing this

culture system, we have established a model which is responsive to the effects of subtle modifications to the environment in vitro. With the ability to examine the cell and molecular events of differentiation from purified, culture-expanded multipotent MSCs, we have the capacity to address experimental questions which cannot be answered using more mature and heterogeneous human osteoblasts derived from trabecular bone explants. The recent development of serum-free defined media for rat and human MSC cultivation [Lennon et al., 1995; J. Holecek, D. Lennon, S. Haynesworth, A. Caplan, and D. Marshak, personal communication] will further facilitate examination of the effects of known bioactive factors, provide a useful experimental platform for identifying new bioactive factors, and assist in the ultimate characterization of their mechanisms of action. Such an advanced understanding of the cellular and molecular events of bone formation may, in the future, allow us to control human MSC differentiation in situ to treat various pathologic conditions resulting from insufficient osteoblastic activity or cell number.

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LABORATORY INVESTIGATION

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Human Vasculogenesis Ex Vivo: Embryonal Aorta as a Tool for Isolation of Endothelial Cell Progenitors

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SUMMARY: Vasculogenesis, the de novo formation of new blood vessels from undifferentiated precursor cells or angioblasts, has been studied with experimental in vivo and ex vivo animal models, but its mechanism is poorly understood, particularly in humans. We used the aortic ring assay to investigate the angioforming capacity of aortic explants from 11- to 12-week-old human embryos. After being embedded in collagen gels, the aorta rings produced branching capillary-like structures formed by mesenchymal spindle cells that lined a capillary-like lumen and expressed markers of endothelial differentiation (CD31, CD34, von Willebrand factor [vWF], and fms-like tyrosine kinase-1 [Flk-1]/vascular endothelial growth factor receptor 2 [VEGFR2]). The cell linings of these structures showed ultrastructural evidence of endothelial differentiation. The neovascular proliferation occurred primarily in the outer aspects of aortic rings, thus suggesting that the new vessels mainly arose from immature endothelial precursor cells localized in the outer layer of the aortic stroma, ie, a process of vasculogenesis rather than angiogenesis. The undifferentiated mesenchymal cells (CD34+/CD31−), isolated and cultured on collagen-fibronectin, differentiated into endothelial cells expressing CD31 and vWF. Furthermore, the CD34+/CD31+ cells were capable of forming a network of capillary-like structures when cultured on Matrigel. This is the first reported study showing the ex vivo formation of human microvessels by vasculogenesis. Our findings indicate that the human embryonic aorta is a rich source of CD34+/CD31− endothelial progenitor cells (angioblasts), and this information may prove valuable in studies of vascular regeneration and tissue bioengineering. (*Lab Invest 2001, 81:875–885*).

Angiogenesis (the sprouting of new blood vessels from the differentiated endothelium of pre-existing vessels) plays an important role in physiologic and pathologic processes (Folkman, 1985). Angiogenesis occurs *normally* during embryonic development, tissue regeneration, and the menstrual cycle and *pathologically* in cancer, proliferative retinopathy, and rheumatoid arthritis (Folkman and Shing, 1992). In contrast, vasculogenesis (the de novo formation of new blood vessels from undifferentiated precursor cells or angioblasts) occurs mainly during embryonic development (Risau, 1995). Vasculogenesis has been studied with experimental in vivo and ex vivo animal models, but its mechanisms, particularly in humans, are poorly understood (LeDourain, 1973; Pardanaud et al, 1987; Risau and Flamme, 1995). In an attempt to fill this gap, we used the aortic ring assay developed by Nicosia (Nicosia and Ottinetti, 1990; Nicosia et al, 1982) to investigate the angioforming capacity of aortic explants from human embryos and the role of vasculogenesis in this process.

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Results

Outgrowth of Vascular-Like Cords from Aorta Ring Explants

Aortic ring explants from 11- to 12-day-old embryos embedded in collagen gels (Elsdale and Bard, 1972; Nicosia and Ottinetti, 1990) were observed for up to 10 days. During the first 24 hours, there were no significant changes, although a few spindly fibroblast-like cells migrated into the matrix. Over the subsequent 24 hours, cohesive cellular cords began to sprout from the aortic rings (Fig. 1, a, b, and c). The number of cord-like structures was highly variable and difficult to quantify because of the complexity of the three-dimensional outgrowth and abundant fibroblast-like cells. Few cords appeared to arise from the cut edges

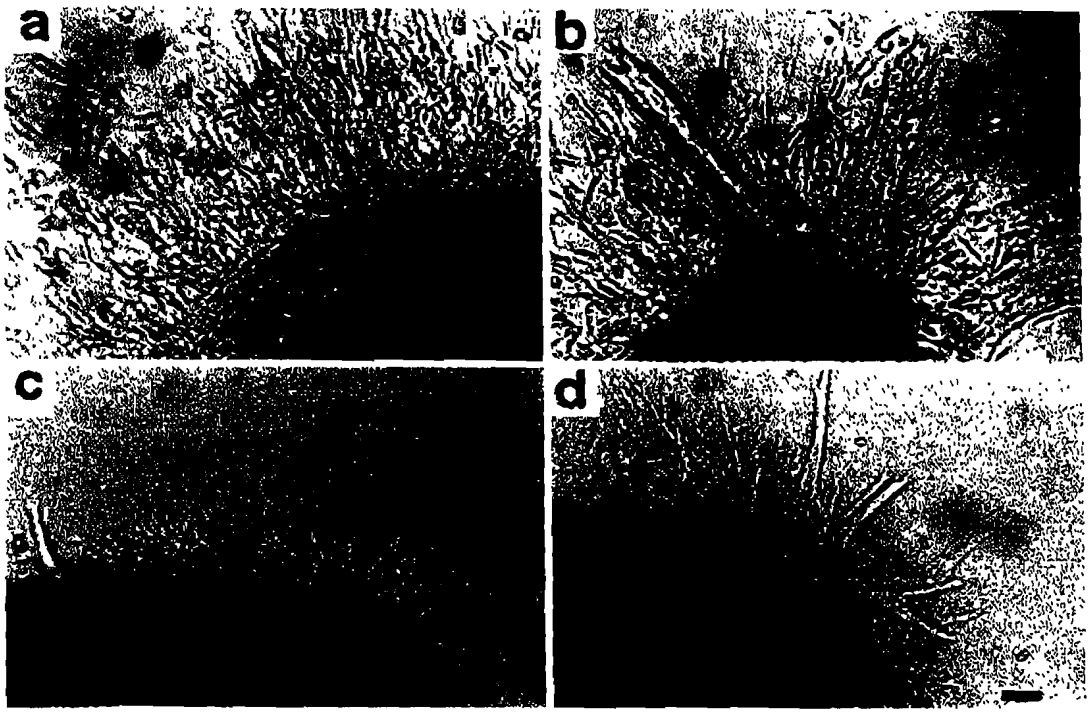

Figure 1.

Micrographs of collagen gel cultures of various human embryonic aorta explants. Vascular outgrowths arising from them are shown at different times of culture. a, A single capillary-like structure surrounded by spindly mesenchymal cells after 48 hours of culture. b, Three large cords arising from the same area of an aortic explant, photographed after 48 hours of culture. c, Incipient buds emerging from a wide area of an aortic explant cultured in collagen for 48 hours. d, Outgrowths of branching capillary-like tubes after 72 hours of culture. The cords grow haphazardly and divide into branches to form a more complex arborizing pattern than at the earlier stages seen in Panels a, b, and c. Scale bars: 25 μm (a, b), 50 μm (c, d).

of the explant, whereas most emerged from adventitial or intimal surfaces. By the third day, the cords had grown haphazardly, dividing into branches and forming complex arborizing patterns (Fig. 1d). At this time some of the newly formed cords had regressed, whereas others were still forming and developing into capillary-like structures. Maximum cord elongation (2–3 mm) was observed after five days. This was followed by rapid regression, which was complete toward the end of the week. These findings were recorded in 70% of the cultures (n = 15).

Characterization of Embryonic Aorta

Histological and immunohistochemical analyses were conducted to characterize embryonic aortic tissue before the in vitro culture in collagen gel. The embryonic aorta wall consisted of a well-formed endothelial lining, an internal elastic lamina, and several external layers of spindly mesenchymal cells. These cells were arranged in compact fascicles and had ultrastructural features of poorly differentiated smooth muscle, including small bundles of myofilaments and surrounding elastic fibers (Fig. 2). At this time of embryonic development, vasa vasorum were absent (Gilbert, 1997).

Immunohistochemistry showed that only the cells lining the lumen stained strongly for CD31 (Fig. 2a) and von Willebrand factor (vWF) (data not shown), thus confirming their mature endothelial phenotype. None of the other cells constituting the aortic wall presented a specific immunoreactivity for these antibodies. The staining with CD34, an antigen typically expressed in endothelial progenitor cells (Flamme and Risau, 1992; Weiss and Orkin, 1996), clearly showed that not only the lumen, but also the more peripheral/para-aortic cells, expressed this antigen. In a serial section of the same explant used for CD31 analysis, CD34 seems to be expressed by the para-aortic tissue (Fig. 2b), whereas, in several sections of different rings, it seems to be constitutively and specifically expressed by the external cells of the aortic wall (Fig. 2c), suggesting that it is not, or not only, a para-aortic cell feature. The external cell layer furthermore showed a strong reactivity with antibody for fms-like tyrosine kinase-1 (Flk-1)/vascular endothelial growth factor receptor 2 (VEGFR2), another marker of endothelial immaturity (Hirashima et al, 1999; Yamashita et al, 2000), which was also expressed by endothelial cells lining the lumen (Fig. 2d).

Characterization of Vascular-Like Cords

The cellular phenotype of cords and capillary tubes arising from aortic rings was investigated by light microscopy, immunohistochemistry, and electron mi-

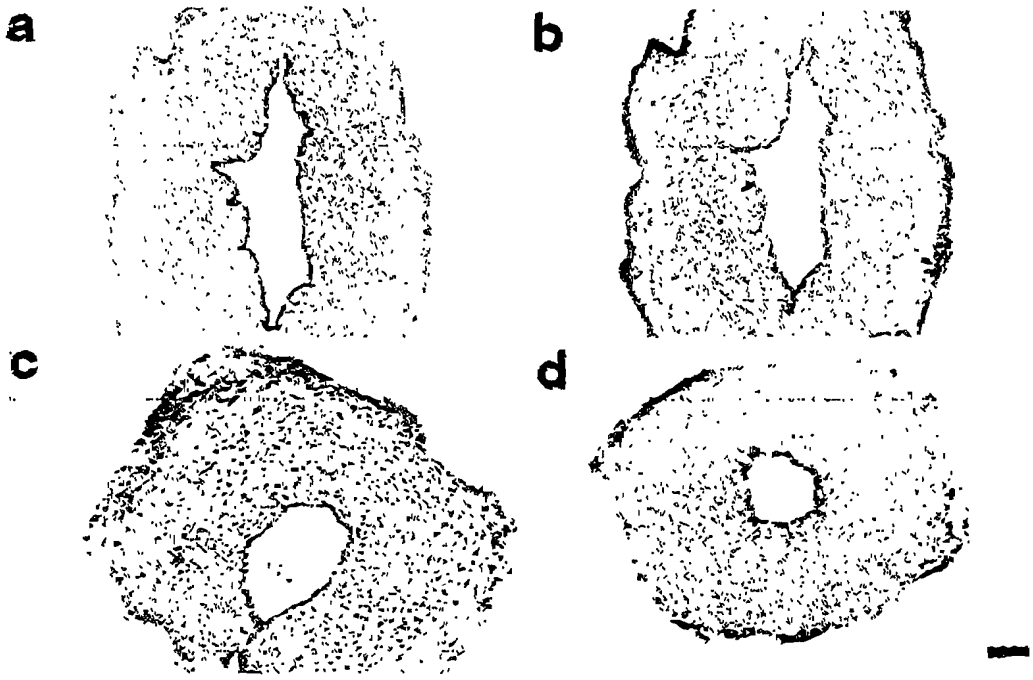

Figure 2.

Immunohistochemical characterization of untreated human embryonic aortas. The tissues were immediately fixed after recovery and then embedded in paraffin and cross-sectioned. a, Aortic cross-section stained for CD31 antigen, showing that only the endothelial cells lining the lumen presented a strong and clear immunoreactivity for this antibody. The other cells constituting the aortic wall were totally negative. b and c, Two representative sections of different explants immunostained for CD34. b, Serial cross-section of the same aortic explant shown in Panel a. Not only did the endothelial cells lining the lumen display an intense positive staining for CD34 antibody, but so also did the more peripheral cell layer that, in some sections, seemed to be a para-aortic tissue (b), in some others, a constitutive layer of cells in the aortic external wall (c). d, The same layers of cells that positively stained for CD34 in Panels b and c also expressed fms-like tyrosine kinase-1 (Flk-1), as shown by the immunohistochemistry conducted on a different tissue section. Scale bar: 160 μm.

croscopy. The outgrowths consisted of mesenchymal spindle cells, sometimes forming aggregates with central necrotic cores (Fig. 3a). Incipient formation of capillary-like structures was often evident in areas where mesenchymal cells were densely packed (Fig. 3, b and c). Cohesive cells with abundant cytoplasm and prominent nuclei lined the capillary-type lumen of these outgrowths. These endothelial-like cells tended to form delicate networks of long straight channels that sometimes branched at an acute angle (Fig. 4a).

Immunochemistry showed that the cells lining the channels stained strongly for CD31 (Fig. 4, a and b) and CD34 (Fig. 4c). They were also immunoreactive for vWF, though less strongly (Fig. 4d). The surrounding mesenchymal cells, which were not organized in vascular structures, were consistently negative for all these markers.

Moreover, the cells forming neovessels, as well as the endothelial cells lining the lumen, stained strongly for Flk-1/VEGFR-2, as demonstrated by different magnification in Figure 4, e and f. Interestingly, Figure 4e shows one of the rare cases in which the microvessels seem to originate both from the internal endothelial layer and the periphery of the aortic section. This phenomenon was recorded in approximately 1 of 20 sections analyzed. In an elevated number of histological sections, in fact, the most of neovascular proliferation occurred primarily in the outer aspects of the aortic rings (Fig. 4a), whereas, in a very limited number of cases, it seemed to originate in the endothelial lining of the aortic lumen (Fig. 4e).

These observations, together with the characteristics of the untreated embryonic aorta (Fig. 2), therefore suggest that neovessels arose mainly from immature endothelial precursor cells (CD34+/Flk1+) of the aortic external mesenchyme, ie, that vasculogenesis rather than angiogenesis was the main process responsible for the vascular outgrowth in these cultures.

By electron microscopy, the aortic outgrowth was found to be composed of an admixture of primitive mesenchymal cells (Fig. 5, a and b), endothelial-lined neovessels (Fig. 5, c and d), and cells with a mixed mesenchymal/endothelial phenotype suggesting differentiation of the mesenchyme into endothelium (Fig. 5b). The lining of the neovessels was composed of differentiated endothelial cells connected by junctional complexes. Endothelial cells exhibited a well-defined luminal/abluminal polarity and rested on a thin and discontinuous basal lamina. The endothelial cytoplasm contained abundant rough endoplasmic reticulum (RER) with focally dilated cisternae, Golgi complexes, pinocytotic vesicles, mitochondria, free ribosomes, and secondary lysosomes including osmiophilic myelin figures (Fig. 5, c and d). Immature

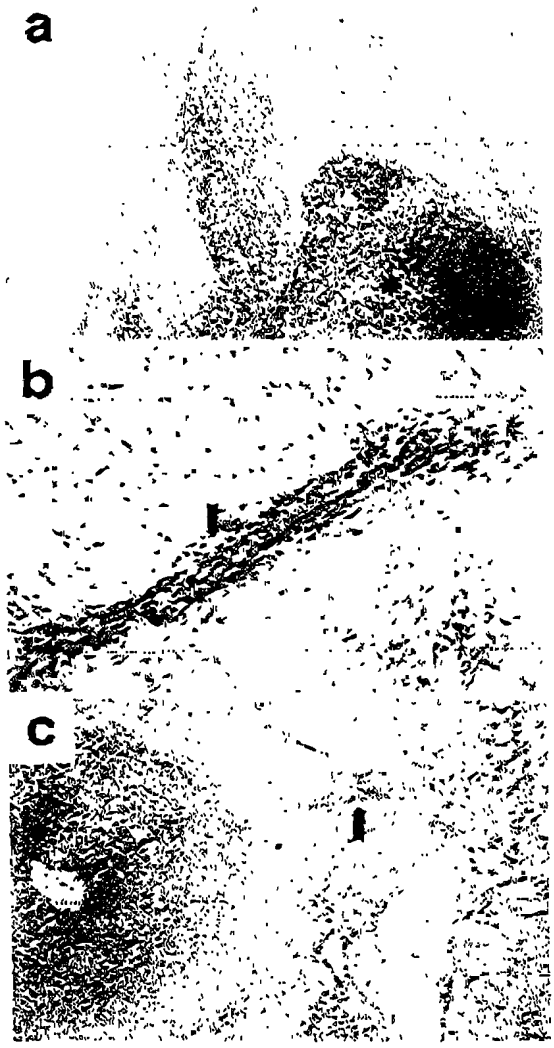

Figure 3.
Light microscopy of capillary-like structures sprouting from an aortic ring. a, Transverse section through an irregular bundle of densely packed mesenchymal cells with central necrotic area (*). b, Longitudinal section of digitiform mesenchymal outgrowth showing central mass of cells at an early stage of differentiation into capillary-like structures (*arrow*). c, Loosely organized proliferation of mesenchymal cells with developing capillary-like structure (*arrow*). This large cord may be an immature vessel structure and studies using a time-lapse recorder are in progress to better evaluate the origin. Scale bars: 100 μm (a, c), 50 μm (b).

mesenchymal cells contained abundant glycogen, mitochondria, and bundles of microfilaments with fusiform densities, which were particularly noticeable in subplasmalemmal locations (Fig. 5, a and b). Cells with a transitional phenotype between mesenchymal cells and endothelium tended to align in longitudinal arrays and establish junctional connections with one another. This caused the separation of newly formed luminal spaces from the surrounding extracellular matrix (Fig. 5b). Cells sequestered within vascular lumina as a result of these morphogenetic changes lost their anchorages to the surrounding matrix and died, leaving behind cytoplasmic debris that was eventually found within differentiated neovessels (Fig. 5c).

Isolation of CD34+ Precursor-Endothelial Cells

To isolate immature endothelial cells (CD34+Flk1+/CD31−) present in the aortic wall, freshly dissected aortas were digested with collagenase-dispase solution. The resulting cell suspension was incubated first with CD31 antibody-coated magnetic beads to remove differentiated endothelial cells and then with beads coated with an antibody against CD34. This procedure demonstrated that less than 1% of cells were CD31-positive, whereas 29.5 ± 2.5% of the cells were CD34-positive (average 1.2×10^6 cells isolated per aorta, $n = 4$). This result was confirmed by flow cytometry analysis (FACS) (Fig. 6).

To better characterize these cells at the time of their isolation, some of them were plated onto dishes coated with collagen-fibronectin (Asahara et al, 1997) and cultured for a short time (24 hours) to avoid differentiation in endothelial basal medium (EBM) supplemented with 10% FCS and endothelial cell-growth supplement (Alessandri et al, 1998). The day after plating, these cells were fixed and stained with CD31 and vWF antibodies not showing immunoreactivity (Fig. 7a).

Furthermore, to determine whether selected CD34+/CD31− cells could differentiate into mature endothelium, they were seeded and cultured for 7 to 10 days in the same conditions described above. The cells were then detached with trypsin and incubated with anti-CD31–coated beads. As a result of this treatment, 25 ± 6.5% (mean of five experiments) of the initial CD34+/CD31− cell population differentiated into CD34+/CD31+ cells (Table 1) which also stained for CD31 and vWF by immunofluorescence (Fig. 7, b and c), thus demonstrating the formation of a more mature endothelial phenotype. Under the same culture conditions, none of the CD31−/CD34− cells differentiated into CD34+/CD31+ cells, nor presented the acquisition of specific immunoreactivity for vWF (data not shown). To further confirm the process of maturation, the CD34+ cells were cultured for 2 to 3 weeks and then seeded on Matrigel to evaluate their capability to form capillary-like tubular structures. In addition to the mature endothelial cells (CD31+) isolated from the aortic lumen and cultured under the same conditions, the CD34+/CD31+ cells were able to form a net of capillary-like structures after 24 hours of incubation (Fig. 7d).

Discussion

Despite the recent substantial increase in our understanding of the molecular mechanisms regulating embryonic vasculogenesis and angiogenesis, there is still much that needs to be learned, particularly in relation to humans. In an attempt to fill a part of this gap, we used the aortic ring assay (Nicosia and Ottinetti, 1990)

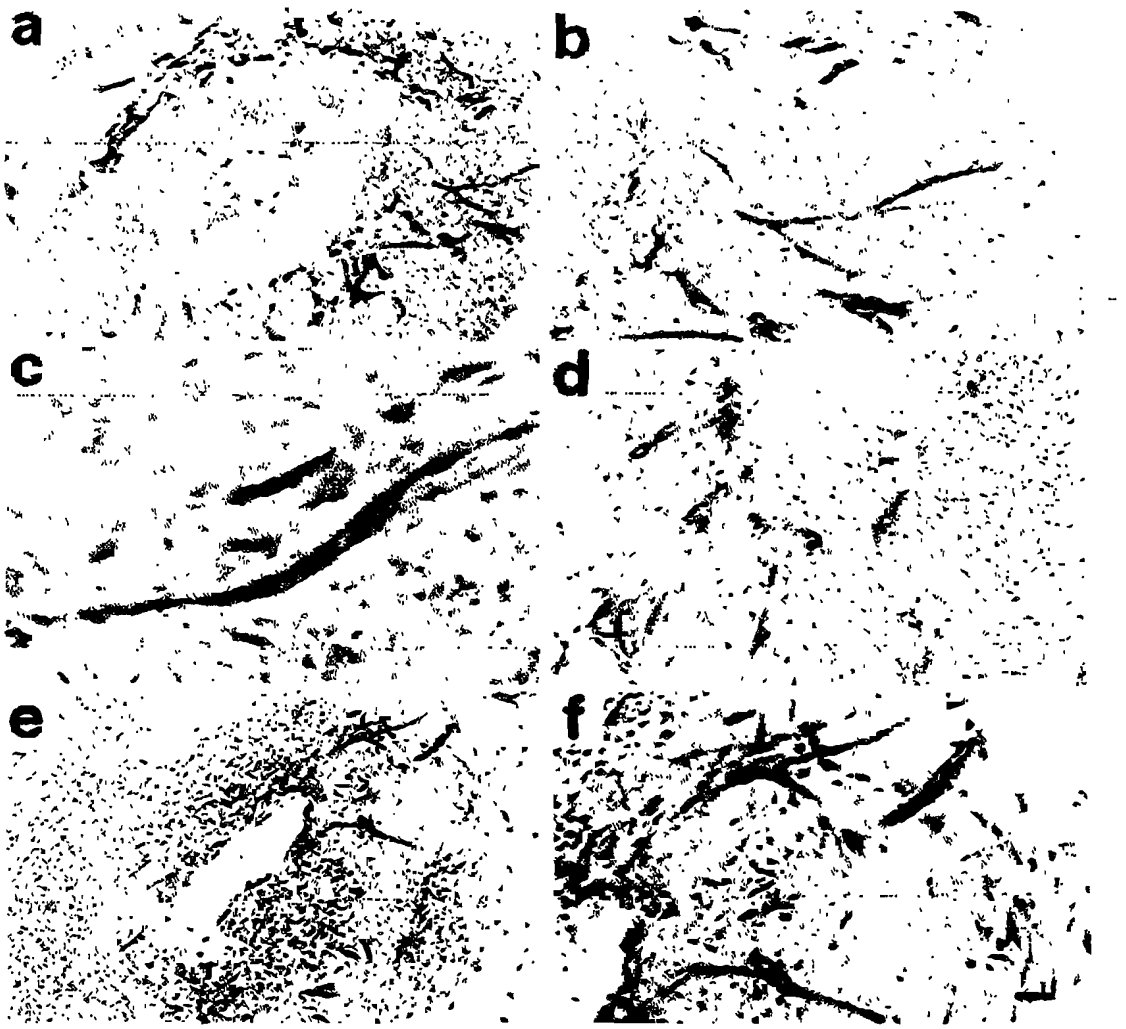

Figure 4.

Immunohistochemistry of capillary-like structures arising from different aortic rings paraffin-embedded after 72 to 96 hours of culture in collagen matrix. a, Cross-section of an aortic ring showing many CD31-positive cells organized into capillary-like channels. These outgrowths appear to arise from a mass of undifferentiated mesenchymal cells at the periphery of the explant. b, Higher magnification of a selected area of the same explant represented in Panel a showing the formation of long and branched channels. c, Capillary-like structures photographed at high magnification showing intense CD34 positivity; surrounding mesenchymal cells are negative. d, Cells of incipient capillary-like channels within a mass of undifferentiated mesenchymal cells are immunoreactive for von Willebrand factor (vWF). e and f, Cross-section of an aortic ring photographed at different magnification showing Flk-1 expression by endothelial cells lining the lumen and by cells organized to form vascular-like channels. In this rare case, the vascular structures seem to originate from the internal layer of endothelial cells. Scale bars: 100 μm (a, e), 50 μm (b, d, f), 25 μm (c)

to analyze the vasculogenesis properties of 11- to 12-week-old human embryonic aortae.

In brief, the results of this study showed that human embryonic aortic explants produce an intense outgrowth of capillary structures when cultured in a three-dimensional collagen matrix, the in vitro formation of which resembles the in vivo physiologic process of embryonic vasculogenesis. Our immunohistochemical and ultrastructural findings suggest that these outgrowths mainly arise as a result of the differentiation of immature CD34+ cells and not from pre-existing endothelial cells (CD31+), although this latter process cannot be entirely excluded.

In fact, as shown by immunohistological data, immature CD34+ cells also express Flk-1/VEGFR-2, another marker highly expressed on the endothelial progenitors cells (Asahara et al, 1999; Hirashima et al, 1999; Peichev et al, 2000). They are spatially localized in the more external mesenchymal layer of cells constituting the aortic wall, in strict correlation with the para-aortic tissue, which however does not contain mature endothelial cells at this stage of development, as demonstrated by their negative staining for CD31.

To the best of our knowledge, this is the first report describing the ex vivo formation of human microvessels by vasculogenesis. A previous study conducted

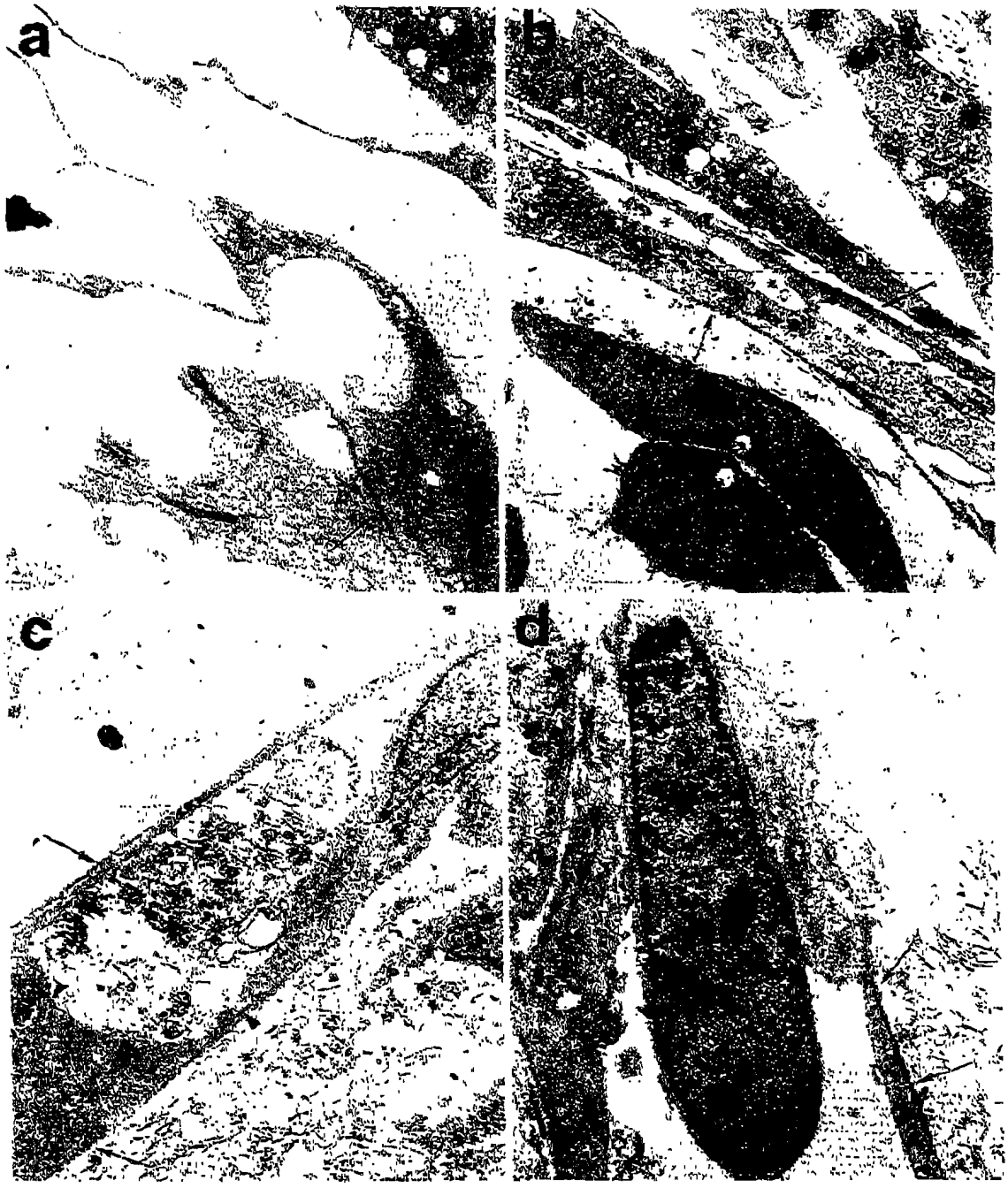

Figure 5.
Electron micrographs of cells from human embryonic aortic ring outgrowths. a, Primitive mesenchymal cells with dendritic cytoplasmic processes and occasional junctional complexes (*arrow*) (×3000). b, Mesenchymal outgrowths showing focal longitudinal alignment of mesenchymal cells into cord-like structure (*arrows*). The cells, connected by junctional complexes (*arrowheads*), have surrounded a space free of extracellular matrix, which may represent a primitive lumen (*asterisks*) (×3000). c, Neovessel lined by endothelial cells connected by junctional complexes (*arrowheads*). The lumen contains cell debris. Endothelial cells rest on a thin and discontinuous basal lamina (*arrows*) (×3000). d, Neovessel surrounded by discontinuous basal lamina (*arrow*) and collagen fibrils. The endothelium exhibits prominent pinocytotic activity, junctional complexes, and well-defined luminal/abluminal polarity. The lumina space is marked by an asterisk (×3000).

by other authors on fragments of fetal human placenta vessels did not investigate this origin of new capillary formation (Brown et al, 1996).

Our data also indicate that the process of endothelial cell (EC) differentiation in humans is different from that using mouse embryonic stem cells (Balconi et al,

Human embryonic aorta cell population: cytofluorimetryc analysis for CD31 and CD34 antigen expression

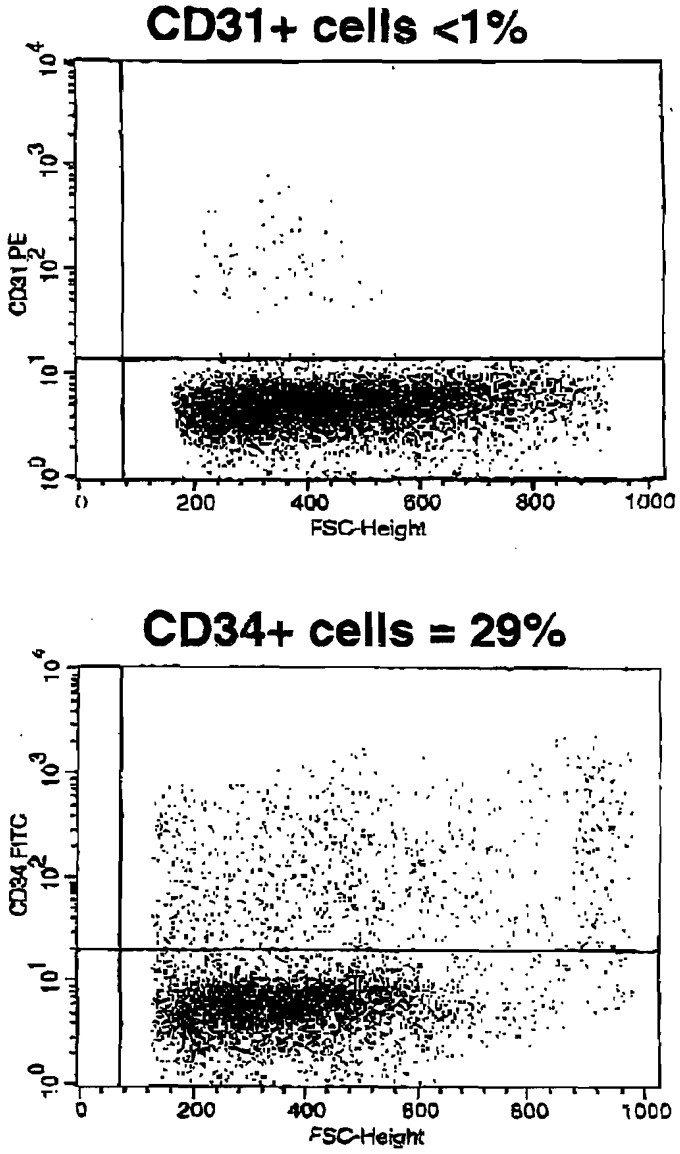

Figure 6.
Binding of anti-CD31 and anti-CD34 antibodies on total cell population obtained by enzymatic digestion of an aortic fragment, as measured by flow cytometry analysis (FACS). Binding of anti-CD31 antibody (upper panel) revealed that a small percentage of cells, approximately 1%, express this antigen, according to the fact that only the cells lining the lumen present a mature endothelial phenotype. The same aortic cell suspension stained with CD34 antibody (lower panel) shows a higher percentage of immunoreactive cells (about 30%)

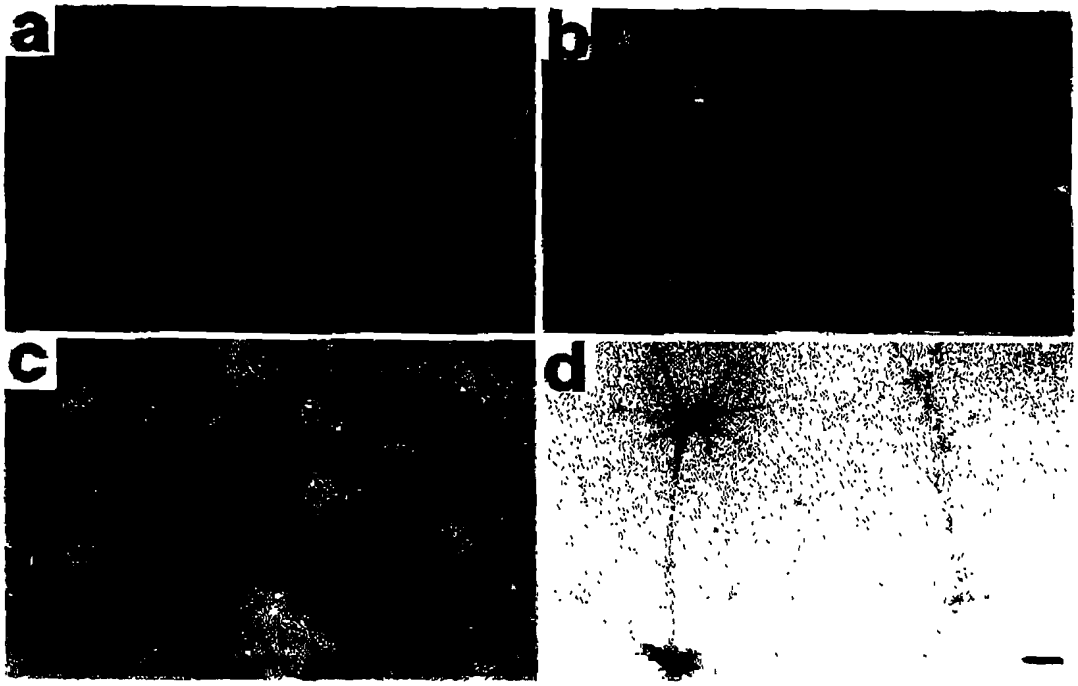

Figure 7.

Immunofluorescence staining of CD34+ cells and formation of capillary structures on Matrigel. Representative culture of CD34+ cells after 10 days of growth on collagen-fibronectin–coated chamber slide stained with anti-CD31(b) and anti-vWF (c). a, CD31 staining performed on CD34+ cells 24 hours after plating revealed no signal expression. At the same time in culture, vWF antigens were also absent. b, Light CD31 expression along the cell-cell contact borders. At the time of culture, usually 20% to 30% of CD34+ cells stained positively for CD31. c, Typical granular perinuclear positivity for vWF staining on CD34+ cells. Its signal intensity and distribution, but not its expression, are comparable to mature endothelial cells isolated from the adult human aorta (data not shown), confirming the immature endothelial phenotype of CD34+ cells. d, Capillary structures formed by CD34+/CD31+ cells 24 hours after seeding on Matrigel. The CD34+/CD31+ cells form tube-like structures, with long vessel-like extensions interconnecting occasional clumps of cells. Scale bars: 17 μm (a, b, c), 250 μm (d).

Table 1. Differentiation of CD34+CD31− Cells Into CD34+CD31+ Cells after 10 Days of Culture

DAY 0		DAY 10	
Antigen selections	% of positive cells	Antigen expressions	% of positive cells
CD34+CD31+	1.3 ± 0.5	**CD34+CD31+**	**25 ± 6.5**
CD34+CD31−	**29.5 ± 2.5**		
CD34−CD31−	60.5 ± 7	CD34+CD31−	69 ± 8.3

2000; Yamashita et al, 2000). Indeed, in contrast to mouse stem cells, the acquisition of CD31 antigen in human vasculogenesis occurs after the maturation of CD34+ cells in culture. Furthermore, our findings indicate that the human embryonic aorta is a rich source of CD34+/CD31− endothelial progenitor cells (angioblasts), which are localized all along the external layer of the aorta mesenchyme, probably in close contact with the para-aortic tissue, which at this stage of development does not contain mature endothelium. We found that only 20% to 30% of the CD34+ cells isolated from aorta give rise to more mature endothelium. The rest of CD34+ cells have not yet been investigated. A recent report of an investigation using mouse embryonic stem cells (Yamashita et al, 2000) suggests that endothelial and mural cells (pericytes and vascular smooth muscle) may originate from the same Flk-1+ precursors, but we do not know if this process occurs also in humans. However, we suggest that among embryonic aorta, it may be possible to find and isolate primitive CD34+ Flk-1+ cells that may differentiate into various vascular cell phenotypes. This may be an important new finding because it could lead to the isolation of vascular stem cells, and a number of previously published reports (reviewed by Fuchs and Segre, 2000) have indicated the enormous potential of multipotent stem cells in clinical applications.

Given the strict correlation between the formation and organization of the hematopoietic/endothelial (Nishikawa et al, 1998) and nervous systems, the possibility of combining the transplantation of angio-

blasts/vascular stem cells and neural stem cells in neurodegenerative diseases represents an appealing approach toward improving the success of this innovative therapy. Furthermore, angioblasts/vascular stem cells could be used to investigate the molecular mechanism involved in human endothelial cell maturation and vasculogenesis, and our model may therefore also be valuable for vascular regeneration studies. Finally, these cells offer an important alternative for the clinical treatment of ischemia and other vascular diseases and suggest possibilities for tissue bioengineering (Niklason, 1999) and gene therapy (Mulligan, 1993).

Materials and Methods

Preparation of Vascular Explants

Permission to use human material was obtained from the ethical committees of the Neurological Institute "C. Besta" and the Institute for Obstetrics and Gynaecology "L. Mangiagalli," Milan, Italy. The tissue was obtained following the ethical guidelines of the Network for European CNS Transplantation and Restoration (NECTAR), which is available online at www.nesu.mphy.lu.se/nectar.

Aortas from normal embryos that were legally aborted were obtained at the Obstetrics and Gynecology Department of L. Mangiagalli Hospital, Milan, Italy. The embryonic aortas were washed several times with PBS and cleaned, care being taken not to damage the vessel walls. Under a dissecting microscope, 1-mm-thick rings were prepared and stored in DMEM at 4° C for not more than 2 hours before use.

The explants were placed in collagen gel using a published procedure with minor modifications (Nicosia and Ottinetti, 1990). Briefly, 7 vol of type I collagen solution (4 mg/ml) prepared from rat tail (Elsdale and Bard, 1972), on ice, was mixed with 2 vol of endothelial basal medium (EBM) (BioWhittaker, Walkersville, Maryland), 5× normal strength, and 1 vol of HEPES (0.2 M). Each well of a 12 multiwell plate was filled with 0.7 ml of collagen solution and left to gel in a humidified incubator at 37° C for 1 hour. One ring per well was placed on top of the gel and a further 0.5 ml of collagen solution added to cover the material. After the second collagen layer had gelled, 1 to 2 hours later, 1 ml of EBM growth medium (Alessandri et al, 1998) was added. The plates were incubated for up to 10 days. The medium was changed every 2 days.

Isolation of Endothelial CD34+/CD31− Cells

After removal, a section of aorta was rinsed several times with PBS, minced rapidly with scissors, and incubated overnight with 0.25% (w/v) collagenase-dispase solution (Boehringer, Mannheim, Germany). After centrifugation, approximately 1×10^5 cells were used for FACS analysis (CD31 and CD34 expression), and the remaining cells were incubated with magnetic beads coated with antibody to CD31 (DAKO, Carpinteria, California) (cell:bead ratio, 1:1). The CD31-negative cells were recovered by centrifugation and further incubated with magnetic beads coated with antibody to CD34 (Dynal, Oslo, Norway). The CD34+/CD31− cells were recovered using a magnetic particle concentrator (Dynal, Oslo, Norway) and cultured on collagen type I (5 $\mu g/cm^2$) and human fibronectin (1 $\mu g/cm^2$) (Boehringer, Mannheim, Germany) coated glass chamber slides (Nunc, Naperville, Illinois), in the presence of EBM growth medium (Alessandri et al, 1998).

Immunocytochemistry of CD34+/CD31− Cells

Twenty-four hours and 7 to 10 days after plating on collagen-fibronectin substrata, the selected CD34+/CD31− cells were fixed in cold 4% paraformaldehyde in PBS, pH 7.4, for 10 minutes at room temperature. They were then washed twice with PBS (0.1% Triton-X, if necessary), blocked with 10% normal goat serum (NGS) (Gibco, Grand Island, New York) and incubated with mouse anti-human CD31 and rabbit antiserum to human factor VIII (respectively, 1:100 and 1:80) (Sigma, St. Louis, Missouri) for 90 minutes at 37° C. After two washings with PBS, the cells were incubated, respectively, with a 1:300 dilution of cyanine dye-labeled goat anti-mouse and anti-rabbit immunoglobulin G (IgG) (Cy2; Jackson Immunoresearch, West Grove, Pennsylvania) for 45 minutes at room temperature. Air-dried cells were then mounted with Fluorsave (Calbiochem, La Jolla, California) and photographed using a Zeiss Axiophot-2-microscope (Oberkochen, Germany). To exclude false positives produced by nonspecific binding of the secondary antibody, the same cells were stained in a similar manner with buffer substituting for primary antibody. Cells that were negative for the beads-selection (CD34−/CD31−) were used as negative controls.

Flow Cytometry Analysis on Freshly Digested Aorta

FACS was performed on the total mixed cell population obtained from collagenase-dispase digestion of an aortic fragment. Approximately 1×10^5 cells were collected, double-stained, and incubated in the dark for 30 minutes at 4° C with anti-CD34, FITC, and anti-CD31 PE (Becton Dickinson, San Jose, California) (dilution 1:10). The cells were then washed twice with PBS and analyzed for fluorescence by flow cytometry using a FACScan (Becton Dickinson, Mountain View, California).

Histology and Immunohistochemistry

Formalin-fixed tissues were included in paraffin following standard histology techniques. Four-micrometer serial sections were transferred to glass slides coated with poly-lysine and rehydrated by immersion in 100% xylene and in a graded ethanol series (100%, 95%, 90%, 80%, and 70%). Sections were then heat treated in a microwave cooker to enhance antigenicity and allow epitope unmasking: twice for 5 minutes each in 1 mM EDTA, pH 8, for CD31, CD34, and vWF antigens, and three times for 4 minutes each in 0.01 M buffer citrate, pH 6, for the Flk-1 antigen.

Endogenous peroxidases were inhibited for 15 minutes at room temperature (RT) with 3% hydrogen peroxide. Samples were then blocked for 20 minutes with 20% normal blocking serum, and the appropriate mixture of primary antibodies was subsequently added to serial sections: mouse anti-CD31 (clone JC/70A, DAKO), diluted 1:10, 30 minutes at RT; mouse anti-CD34 (Serotec, Raleigh, North Carolina), diluted 1:50, 30 minutes at 37° C; rabbit anti-vWF (DAKO), diluted 1:20, 30 minutes at RT; and mouse anti-Flk-1 (Santa Cruz Biotechnology, Santa Cruz, California), diluted 1:50, 45 minutes at RT. After washings, sections were incubated for 30 minutes with appropriate secondary antibodies conjugated to biotin and processed according to the avidin/biotin peroxidase complex method with kit reagents (mouse IgG and rabbit IgG Vectastain; Vector Laboratories, Burlingame, California). Peroxidase activity was shown with 3,3'-diaminobenzidine (Menarini-Biogenex, San Ramon, California) in PBS, and counterstaining was performed with hematoxylin-eosin. To exclude false positives produced by nonspecific binding of the secondary antibody, all of the tissues were treated in the same manner with buffer substituting for the primary antibody.

Cord Formation on Matrigel

Two hundred and seventy microliters of Matrigel (12.5 mg/ml) (Becton Dickinson, Bedford, Massachusetts) at 4° C were transferred to prechilled 24-well culture plates using sterile pipette tips that had been cooled to −20° C before use. After gentle agitation to ensure even coating, plates were incubated for 30 minutes at 37° C to allow the Matrigel to solidify. CD34+/CD31− cells were then seeded at a concentration of 6×10^4/well in EBM growth medium (Alessandri et al, 1998). Cord formation was obtained after 24 hours of incubation.

Electron Microscopy

Selected cultured explants were fixed in 2.5% glutaraldehyde immediately after preparation, postfixed in osmium tetroxide, embedded in Epon-Aaraldite, and observed under a Zeiss CEM 902 electron microscope.

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